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NOVEL INHIBITORS OF LIPID PHOSPHATASE SHIP2 AND THEIR EFFECTS ON THE PHOSPHORYLATION OF AKT KINASE

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Type 2 diabetes mellitus (T2DM) has been shown to be associated with hyperglycemia, insulin resistance, hyperinsulinemia and impaired insulin secretion from pancreatic β-cells leading to micro- and macro-vascular complications including multiorgan failures. At the cellular level, the mechanism of insulin resistance is associated with complex PI3K-Akt mediated insulin signaling pathway. Moreover, lipid phosphatase SHIP2 (Src homology 2 domain containing inositol 5-phosphatase 2) plays a vital role as a negative regulator of the insulin signaling pathway downstream of PI3K by hydrolyzing phosphatidylinositol- 3,4,5-trisphosphate (PIP3) into phosphatidylinositol- 3,4- biphosphate (PIP2). Scientific reports have shown that inhibition of SHIP2 activity might improve Akt phosphorylation and thus PI3K-Akt mediated insulin signaling pathway. Considering this, I am interested in the SHIP2 inhibitors with drug like properties such as improved solubility, pharmacokinetic and bioavailability properties with little to no contraindications.

In the present thesis, I have attempted to detect indirectly the capacity of 8 novel small molecule SHIP2 inhibitors, #160, #161, #162, #163, #167B, #170A, #171, #172 for their ability to phosphorylate Akt kinase in L6 myotubes using immunoblotting as a tool and compared data using graphical representation to pick up the best candidate. Two inhibitors, #163 and #170A were further chosen for alamarBlue® cytotoxicity assay. Treatment with #163 did not display direct cytotoxic effects on the myotubes. The viability of myotubes was not affected at low concentrations of #170A, but it started to reduce at concentrations >200 µM. In my study, I came up with #163 and #170A as the best lead candidates for further analysis. In future, more trials need to be performed with these inhibitors. Moreover, there are several other novel small molecule SHIP2 inhibitors identified from chemical library that need to be tested. Briefly, in this thesis, I have first time reported 8 novel small molecule SHIP2 inhibitors which could be a significant step in the discovery of new T2DM drugs for more efficient, cost effective and safe treatment of the disease with least contraindications.

Avainsanat – Nyckelord – Keywords
Insulin resistance, PI3K-Akt mediated insulin signaling pathway, SHIP2, PIP3, PIP2, SHIP2 inhibitors, L6 myotubes

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ABBREVIATIONS USED IN LITERATURE REVIEW

ACC	Acetyl coenzyme A carboxylase
AMPK	AMP activated protein kinase
aPKC	Atypical protein kinase C
AS160	Akt substrate of 160 kDa
CKD	Chronic kidney disease
CVD	Cardiovascular diseases
DC	Dendritic cells
DM	Diabetes mellitus
DN	Diabetic nephropathy
DN SHIP2	Dominant negative forms of SHIP2
DP-BB	Diabetes prone biobreeding
DPP-4	Dipeptidyl peptidase 4
DR	Diabetic retinopathy
EH	Essential hypertension
FOXO	Forkhead family box O
GADA	Glutamic acid decarboxylase antibodies
GDM	Gestational diabetes mellitus
GK	Goto-kakizaki
GLP-1 RA	Glucagon like peptide -1 receptor agonists
GLUT4	Glucose transporter 4
GP	Gastroparesis
GSK3β	Glycogen synthase kinase 3β
HbA1c	Hemoglobin A1c
HOMA	Homeostatic model assessment
IFG	Impaired fasting glycemia
IR	Insulin receptor
IRS	Insulin receptor substrate
IRS-1	Insulin receptor substrate-1
ISA	Insulin sensitizing agents
LADA	Latent autoimmune diabetes in adults
LBDD	Ligand based drug discovery
MALA	Metformin associated lactic acidosis

MAPK	Mitogen activated protein kinase
MARD	Mild age-related diabetes
mGPD	Mitochondrial glycerophosphate dehydrogenase
MOD	Mild obesity related diabetes
MONW	Metabolically obese normal weight
mTOR	Mammalian target of rapamycin
NOD	Non-obese diabetic
PDK1	3-phosphoinositide -dependent protein kinase 1
PI-3,4,5-P3/PIP3	Phosphatidylinositol-3,4,5-triphosphate
PI-3,4-P2/PIP2	Phosphatidylinositol-3,4-biphosphate
PI3K	Phosphatidylinositol (PI) 3-kinase
PI-4,5-P2	Phosphatidylinositol-4,5-biphosphate
PKB	Protein kinase B
PPARs	Peroxisome proliferator activated receptors
PRD	Proline rich domain
PTEN	Phosphatase and tensin homolog on chromosome 10
RABGAP	Rab- GTPase activating protein
SAID	Severe autoimmune diabetes
SAT	Subcutaneous adipose tissue
SGLT2	Sodium glucose cotransporters 2
SHIP2	Src homology 2 domain containing inositol 5-phosphatase 2
SHR	Spontaneously hypertensive rats
SIDD	Severe insulin deficient diabetes
SIRD	Severe insulin resistant diabetes
T1DM	Type1 diabetes mellitus
T2DM	Type2 diabetes mellitus
TBC1D4	RABGAP TBC1 domain family member 4
TSC1/2	Tuberous sclerosis protein 1/2
TZDs	Thiazolidinediones

1. INTRODUCTION

Diabetes mellitus (DM) is one of the major threats to human health worldwide. Insulin-dependent diabetes mellitus (type 1 diabetes mellitus, T1DM) and non-insulin-dependent diabetes mellitus (type 2 diabetes mellitus, T2DM) are the two primary types of DM[1]. In T1DM, autoimmune destruction of β-cells leads to outright deficiency of insulin resulting into hyperglycemia (critically high levels of glucose in bloodstream), whereas T2DM is associated with impaired insulin production and insulin resistance leading to an impaired insulin action in glucose metabolism resulting into hyperglycemia[2, 3, 4]. In 2010, around 285 million people were living with DM[5]. In 2017, this number increased to 425 million people worldwide, 90% of whom were living with T2DM (International Diabetes Federation-IDF, 2017). In low- and middle-income countries, the increase in number was more prominent, and more frequent in men compared to women[6]. In addition to this, another 350 million people were susceptible to develop T2DM in the same year[7]. By 2045, the number of people (20 -79 years of age) with DM is projected to burgeon up to 629 million[5]. Moreover, gestational diabetes mellitus (GDM), which is diagnosed in the second or third trimester of pregnancy, affects large population of pregnant women worldwide[2, 8]. According to the report of IDF in 2017, approximately 14 % live births were affected by GDM.

T2DM is primarily caused by the interplay among genetic risk factors related to compromised insulin secretion and insulin resistance, environmental factors such as obesity, stress, aging, and several other factors[1,9]. Due to insulin resistance and high blood glucose level, T2DM patient is more prone to macro vascular diseases such as hypertension, strokes, heart attack, vascular disease and peripheral vascular diseases, microvascular diseases such as nephropathy, retinopathy and neuropathy, and various forms of cancers[1]. Studies have shown that excessive nutrition in individuals susceptible to metabolic diseases causes peripheral tissues to resist insulin action and thereby, affect the blood glucose uptake which ultimately raises the level of blood glucose. This incident eventually leads to excess secretion of insulin by islet β-cells of pancreas termed as 'hyperinsulinemia'[10,11,12]. However, due to varied experimental observations, there is a debate whether insulin resistance is the one that causes hyperinsulinemia or vice versa. Animal studies have shown that chronic hyperinsulinemia is associated with reduced insulin sensitivity[13, 14,15]. Research in Lepob/ob mice has shown that insulin resistance, obesity and increased lipogenesis are preceded by hyperinsulinemia[15, 16,17]. This clearly shows that T2DM is linked to malicious complex web.

At mechanistic level, the pathophysiology of T2DM and associated insulin resistance depend on a complex chain of phosphatidylinositol (PI) 3-kinase -Akt-mediated insulin signaling pathway in which varied perturbations such as posttranslational modifications and/or mutations in

insulin receptors, insulin receptor substrates (IRS) or in downstream molecules could be impetus behind the impairment in the pathway. Decreased phosphatidylinositol (PI) 3-kinase (PI3K)/Akt activities and impaired expression and function of GLUT4 glucose transporter proteins are the most common modifications caused in insulin resistance[18]. One critical factor that contributes to the disturbance of PI3K-Akt pathway is hyperphosphorylation of IRS proteins at Ser/Thr. It eventually decreases its interaction with PI3K kinase due to decreased phosphorylation at Tyr and ultimately reducing the phosphorylation and activation of Akt kinase[19]. Moreover, lipid phosphatases such as 3' phosphatase PTEN (Phosphatase and tensin homolog on chromosome 10) and 5' phosphatase SHIP2 (the Src homology 2 domain containing inositol 5-phosphatase 2) play vital roles in negatively regulating insulin signaling pathway below PI3K by altering phosphatidylinositol- 3,4,5-trisphosphate (PIP3) levels by hydrolyzing it to phosphatidylinositol-4,5-biphosphate (PI(4,5)P2) or phosphatidylinositol- 3,4- biphosphate (PI(3,4)P2) respectively[20,21,22]. Sasaoka *et al* (2001) have reported that overexpression of SHIP2 inhibits insulin activity via its 5'-phosphatase activity in L6 myotubes, acting as a negative regulator of PI3K-Akt-mediated insulin signaling pathway[23]. SHIP2 has been shown to be upregulated in the skeletal muscle of an insulin-resistant diabetic db/db mice model[20]. Also, insulin sensitizing agents such as Rosiglitazone ameliorate upregulated SHIP2 resulting in insulin induced Akt activation marking the association of SHIP2 with insulin resistance[20].

Since scientific literature has reported the association of SHIP2 with insulin resistance, inhibiting the 5' –phosphatase activity of SHIP2 could offer a new target for the treatment of T2DM with despaired insulin signaling. There are few SHIP2 inhibitors reported in scientific journals in recent years[24,25,26,27,28]. However, due to poor drug like properties of these inhibitors, further investigation is needed to identify inhibitors that can be used as medicinal drugs.

2. BACKGROUND LITERATURE REVIEW

2.1 Diabetes and its classification

Diabetes mellitus (DM) is defined as 'heterogeneous disturbances of metabolism'[29]. Such disturbances are mainly the result of hyperglycemia, possibly leading to diffused vascular impairment and multiorgan failure[30,31]. The cardinal cause for this could be explained by either disturbed insulin secretion or disturbed action of secreted insulin hormone or by both[29]. DM is becoming more and more common health issue at global level and no longer limited to just the western lifestyle. According to American Diabetes Association (ADA), 2014, DM is not a single disease but consisted of several diseases classified by etiology and pathology, such as Type 1 diabetes mellitus (T1DM), Type 2 diabetes mellitus (T2DM), gestational diabetes mellitus (GDM) and the class 'other' which includes monogenic diabetes and cystic fibrosis related diabetes[32,33]. Insulin deficiency is the most common feature in different classes of diabetes syndrome mentioned above[34]. Among varied classes, T2DM affects almost 85-90 % of the population suffering from diabetes, GDM affects ~5-6% of the pregnant females who in most cases show early forms of T2DM, and patients with T1DM account for ~5-10%[32,34]. Since diabetes is heterogeneous and vary significantly, the assigned type of DM to a patient is often misleading since it is based on the circumstances at the time of the diagnosis[2].

Three kinds of complications are associated with diabetes: Macrovascular, microvascular and neurologic. Increased blood glucose is the common denominator of all the three kinds of complications of diabetes. Long-term prognosis of the patients with diabetes is obscure; however, the chances are that the individuals may develop diabetic retinopathy (DR) with possible vision impairment, diabetic nephropathy (DN) resulting in renal failure, and neuropathy[31]. Diabetic patients are frequently diagnosed with cerebrovascular, peripheral arterial and atherosclerotic cardiovascular disease and often suffer from hypertension and impaired lipoprotein metabolism[33]. Early onset atherosclerosis of the coronary arteries is common macrovascular disease among diabetic patients and is frequently found to be the primary cause of death of an individual with diabetes[34].

Numerous risk factors either modifiable, such as life style of an individual, glycemic control, dyslipidemia, hypertension, or non-modifiable such as genomic construction, age, duration of diabetes, are associated with the pathogenesis of diabetes[31]. Hyperglycemia is a crucial determinant in the development of vascular complications of diabetes, both acute and chronic hyperglycemia have lethal effects on diabetic patients[30].

2.1.1 Type 1 Diabetes Mellitus (T1DM)

T1DM is a chronic autoimmune disorder involving the selective destruction of insulin-secreting pancreatic β cells by a β cell–specific autoimmune mechanism. It is one of the most common chronic disorders occurring in children and adolescents, however it can be diagnosed in adults[35]. β-cells are responsible for maintaining glucose level within a narrow physiological range by sensing glucose and secreting insulin. Hence, once these cells are destroyed, the maintenance of blood glucose level is disturbed resulting in ketoacidosis and severe hypoglycemia. Secondary complications such as renal failure, vision loss or heart disease could also occur as consequences. Moreover, T1DM is a multifactorial disease making it difficult to elucidate the pathogenesis of the disease[36,37]. In the progression of T1DM, genetic predisposition is crucial, however, the concordance rate was found to be only ~40% in the studies of T1DM in identical twins indicating the contribution of nongenetic factors in the disease development[38]. To understand the pathogenesis of human T1DM, the non-obese diabetic (NOD) mice models and diabetes prone BioBreeding (DP-BB) rat models have been extensively studied. It has been shown that several different immune cell types such as CD4+ and CD8+ T cells, B lymphocytes, macrophages, dendritic cells (DC) and β cell autoantigens are involved in the β-cell specific autoimmune process[39]. The immunologically activated CD8+ cytotoxic T cells along with activated CD4+ helper T cells destroy β-cells in islets. Moreover, the damage to β-cells is also done by granzymes, perforin, reactive oxygen species and synergistic interactions of cytokines[40].

For T1DM, exogenous insulin administration is quite prominent therapy. However, due to precise dosing issues and number of injections and other issues related with the complexity of the disease, stem cell based β-cell replacement therapies are under consideration for the treatment of T1DM for its cost effectiveness[41].

2.1.2 Type 2 Diabetes Mellitus (T2DM)

T2DM was earlier believed to be an exclusive adult metabolic disease, but, since the beginning of the 21st century, it has been reported more commonly even in youth and adolescents, and occasionally in children[42,43]. It is associated with obesity and overweight. However, metabolically obese normal weight (MONW) individuals with insulin resistance are at higher risk of developing T2DM than overweight individuals without insulin resistance or metabolic disorder. The factors responsible for metabolic obesity in normal weight individual might possibly involve central obesity, low weight at birth, lack of exercise and active routine, and family history[44]. Hyperglycemia and altered lipid metabolism are important characteristics of T2DM resulting from inadequate responses of pancreatic islet β cells and adipose tissue to chronic fuel surfeit[44]. The damaged pancreatic islets and stressed

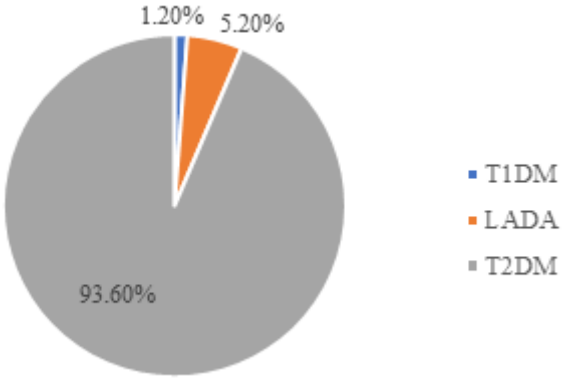
adipose tissues lead to elevated concentrations of inflammatory cytokines in peripheral tissues such as skeletal muscle and liver due to nutrient spillover. Thus, metabolic defects such as damaged islet cells, impairment of subcutaneous adipose tissue (SAT) expansion, enhanced production of endogenous glucose in the liver and peripheral insulin resistance contribute to the development of T2DM[45,46].

Once hyperglycemia is present, insulin resistance is the key predictor of T2DM and could be a target of therapeutic interventions. At the pre-onset of T2DM, resistance to insulin leads to extra secretion of insulin to compensate slightly high concentration of glucose in blood, ultimately leading to hyperinsulinemia. When hyperinsulinemia can no longer compensate insulin resistance, diabetic state persists, and hyperglycemia becomes apparent in fasting and post-prandial state. Hyperglycemia leads to a diffuse endothelial dysfunction, micro and macrovascular complications of diabetes and multiorgan failure[30,31]. Among several mechanisms by which hyperglycemia contributes to vascular complications, polyol pathway is the most crucial mechanism contributing to diabetic complications by increasing glucose flux[47]. Skeletal muscle is the major site of insulin-mediated glucose uptake in the post-prandial state. Impaired glycogen synthesis in muscle tissues plays a crucial role in the development of insulin resistance. There are three rate controlling steps in the metabolism of muscle glucose: GLUT4 glucose transporter, hexokinase, glycogen synthase, which have been implicated in impaired glycogen synthesis. Intracellular impairments in glucose transport is the rate controlling step for glucose uptake in muscle tissues. Impaired intramyocellular fatty acid metabolism could lead to such defects. Through defects in intramyocellular fatty acid metabolism, fatty acids could lead to insulin resistance by activating a serine kinase cascade, leading to decreased insulin-stimulated (IRS)-1 tyrosine phosphorylation and eventually decreased activity of PI3K kinase, which is a key step in insulin-stimulated glucose uptake into muscle[48].

2.1.3 Novel classification of adult-onset DM: recent findings

Ahlqvist *et al* (2018) recently described a new substratification of diabetes based on six variables: glutamic acid decarboxylase antibodies (GADA), age of the patient at the time of diagnosis, HbA_{1c} (Hemoglobin A1c – glycated hemoglobin), BMI (Body Mass Index) and homeostatic model assessment (HOMA) quantifying insulin resistance and pancreatic β cell function. In their study, they used data from four separate populations divided into five cohorts from Sweden and Finland (Figure 1)[48] to substratify diabetes into five clusters[49].

(A) Traditional classification-based distribution of ANDIS cohort



(B) Distribution of ANDIS cohort based on k-means clustering

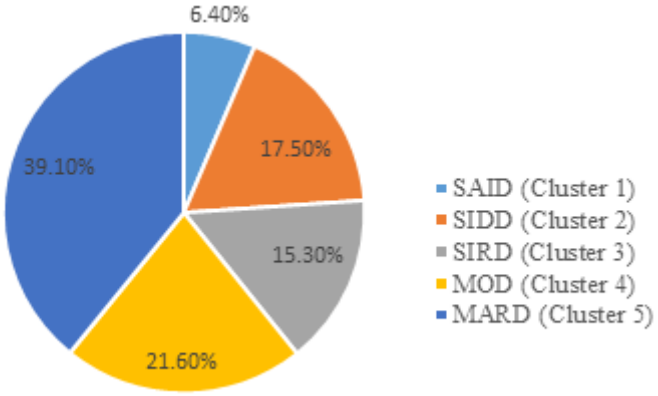


Figure 1. Distribution of patients based on classification method by Ahlqvist *et al* (2018)[49], images modified from the original (License to reuse the image has been obtained from Elsevier)

LADA: latent autoimmune diabetes in adults. SAID: severe autoimmune diabetes. SIDD: severe insulin-deficient diabetes. SIRD: severe insulin-resistant diabetes. MOD: mild obesity-related diabetes. MARD: mild age-related diabetes. ANDIS: All New Diabetics in Scania[49].

Cluster 1 (severe auto immune diabetes – SAID) was associated with poor metabolic control, insulin deficiency, presence of GADA, relatively low BMI and was found to be an early onset disease. Cluster 2 (severe insulin deficient diabetes - SIDD) was an early onset disease with relatively low BMI, poor insulin secretion and poor metabolic regulation. Cluster 3 (severe insulin resistant diabetes – SIRD) was associated with high insulin resistance and high BMI. Cluster 4 (mild obesity related diabetes – MOD) was characterized by obesity. Cluster 5 (mild age-related diabetes – MARD) was a late onset disease. In their study, the patients in cluster 3 were at higher risk of diabetic kidney disease than patients in other clusters, however, the current treatment did not differ in their prescription. Moreover, patients in cluster 2 were most likely to develop retinopathy. Further, they found that a T2DM-associated variant in the locus TCF7L2 was linked to SIDD, MOD and MARD whereas a T1DM-associated variant in the locus HLA (Human Leukocyte Antigen) was strongly linked to

SAID. The researchers could come up with two new severe forms of diabetes: SIDD and SIRD, which were traditionally covered under T2DM[49].

This novel classification may assist in individualized medicine – a branch of medicine that takes into account the genetic, environmental and lifestyle variability of individuals rather than the one-size-fits-all strategy to treat this heterogenous disease. The key advantages are its ability to predict diabetic nephropathy in SIRD and retinopathy in SIDD[49]. The clustering in the study of Ahlqvist *et al* suggests to target insulin resistance in randomized trials and enhance insulin sensitivity in SIRD. However, there are no potential drugs available to enhance insulin sensitivity except pioglitazone[49].

2.2 Insulin Signaling

2.2.1 PI3K/Akt mediated insulin signaling pathway and glucose uptake

Insulin signaling network is complex and has effects on cellular metabolism, growth and differentiation. Muscle tissues, adipose tissues, hepatic tissues and neurons are the major sites of insulin action[50]. Insulin performs its fundamental actions by binding to insulin receptors leading to the activation of three major pathways: (i) The PI3K-Akt pathway responsible for glucose uptake in muscle cells and adipocytes, (ii) The TSC1/2-mTOR pathway important for protein synthesis and cellular energy homeostasis, and (iii) The RAS-MAPK (mitogen activated protein kinase) pathway responsible for cell proliferation, division and motility[51]. Insulin regulates glucose homeostasis by stimulating glucose transport in insulin sensitive skeletal muscle and adipose tissues. Insulin stimulated glucose uptake takes place primarily in skeletal muscle by the translocation of glucose transporters, primarily GLUT4, from intracellular locations to the plasma membrane[52]. The deposited glucose is converted into glycogen by glycogen synthase and stored as glycogen in human muscle fibers[53].

Insulin-dependent translocation of GLUT4 is initiated by insulin binding to insulin receptor (IR) which activates its tyrosine protein kinase-containing intracellular β subunit leading to the receptor autophosphorylation on tyrosine residues[54]. Subsequently, insulin receptor tyrosine kinase phosphorylates insulin receptor substrates – IRS1 and IRS2, leading to the activation of the phosphatidylinositol (PI) 3-kinase (PI3K) mediated insulin signalling pathway[55] (Figure 2). IRS proteins belong to a family of adaptor proteins, which recruit the catalytic subunit of PI3K kinase, play a role in converting the signal of tyrosine phosphorylation to the signal of lipid kinase. Class 1a PI3K is activated through the binding of its SRC homology 2 (SH2)-domains of the p85 regulatory

adaptor subunits to phosphorylated YMXM motifs in IRS-proteins. This results into the activation of the p110 catalytic subunit of PI3K.

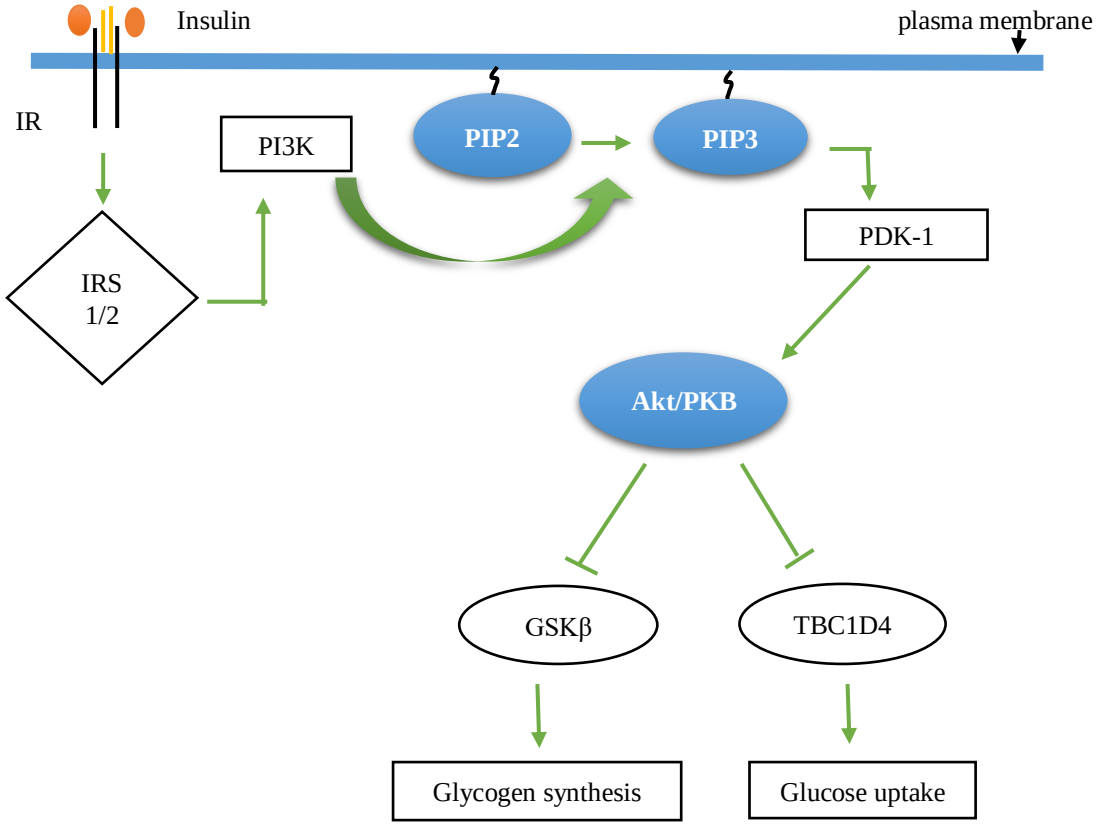


Figure 2. PI3K mediated insulin signalling pathway (simplified)[50].

Once insulin binds to the IR, IR is autophosphorylated and phosphorylates IRS proteins. PI3K associates with phosphotyrosine sites on IRSs. Subsequently, PI3K kinase converts PIP2 into PIP3 at the plasma membrane. This activates PDK1 that phosphorylates Akt at the Thr308. A second phosphorylation takes place at the Ser473 (not shown) by mTOR2. Activated Akt phosphorylates downstream molecules including GSKβ and TBC1D4 mediating the effects of glycogen synthesis and glucose uptake respectively through their inactivation by Akt

Activated PI3K quickly phosphorylates phosphatidylinositol 4,5-bisphosphate (PIP_2) to generate phosphatidylinositol 3,4,5-trisphosphate (PIP_3), which further activates PDK-1 (3-phosphoinositide-dependent protein kinase-1) through its PH domain. Activated PDK1 phosphorylates Akt at Thr-308. For the complete activation of Akt, it is further phosphorylated at Ser-473 by the mammalian target of rapamycin complex 2 (mTORC2). Thus, Akt is recruited to the plasma membrane from the cytosol and activated fully by its phosphorylation at Thr-308 and Ser-473[56]. Activated Akt Ser/Thr kinase phosphorylate downstream substrates such as the forkhead family box O (FOXO) transcription factors; the protein tuberous sclerosis 2 (TSC2), glycogen synthase kinase 3β (GSK3β) and Akt substrate of 160 kDa (AS160), also called TBC1D4 (the RABGAP TBC1 domain family member

4)[50]. Serine phosphorylation of GSK3β by Akt leads to its deactivation resulting into the activation of glycogen synthase and the synthesis of glycogen from glucose[57]. TBC1D4, a Rab-GTPase-activating protein (RABGAP) is the Akt2 substrate responsible for GLUT4 translocation to the plasma membrane. In the unphosphorylated state of TBC1D4, the target RABS are in GDP-bound inactive form. In muscle cells, the target RAB isoform is RAB8a166 whereas in adipocytes, RAB10 is the target of TBC1D4[50]. The phosphorylation of TBC1D4 by Akt kinase leads to the activation of RAB proteins resulting into the docking and fusion of GLUT4 vesicles to the plasma membrane. The recruitment of GLUT-4 vesicles to the plasma membrane is inhibited in an TBC1D4/AS160 mutant lacking Akt phosphorylation sites[58].

2.2.2 Negative regulation of insulin action by lipid phosphatases

2.2.2.1 Phosphatase and tensin homologue on chromosome 10 (PTEN)

In addition to the regulation of PIP3 synthesis and concentration at the plasma membrane, its localization and degradation is regulated by two lipid phosphatases that dephosphorylate PIP3, thus negatively regulating the insulin signalling (Figure 3).

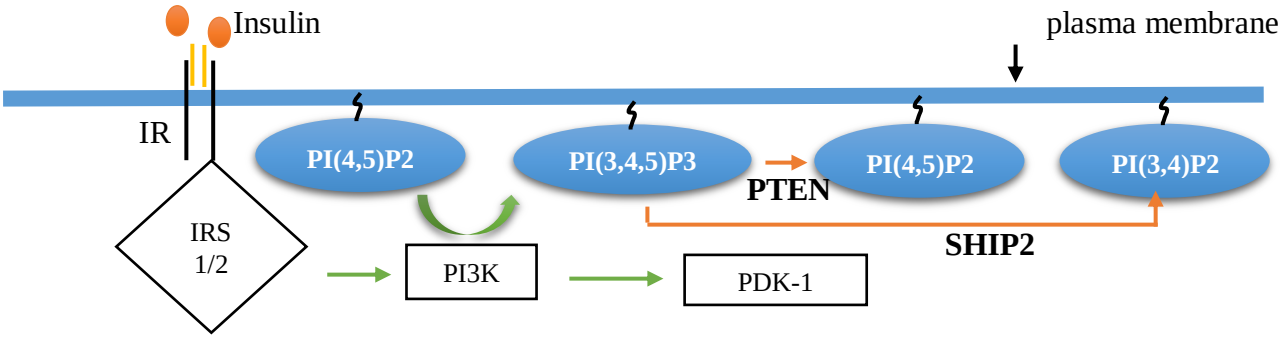


Figure 3. Negative regulation of PI3K mediated insulin signaling[50]: lipid phosphatases PTEN and SHIP2 negatively regulate PI3K-Akt mediated insulin signaling pathway through their 3'- phosphatase and 5'-phosphatase activities respectively.

Phosphatase and tensin homologue on chromosome 10 (PTEN) is 3'-phosphatase that hydrolyses PIP3 into its precursor phosphatidylinositol (4,5)-biphosphate (PI(4,5) P2) and thus it is a negative regulator of the PI3K/Akt signalling pathway[59]. The inhibition of PTEN activity results into enhanced glucose metabolism, cell growth and survival pathway through elevated Akt activity[49]. On the other hand, in 3T3-L1 adipocytes, overexpression of PTEN leads to inhibition of GLUT4 translocation to the plasma membrane and hence restricts glucose uptake[60].

2.2.2.2 Src homology 2 (SH2) domain-containing inositol 5′-phosphatase 2 (SHIP2)

Another ubiquitously expressed lipid phosphatase, Src homology 2 (SH2) domain-containing inositol 5′-phosphatase 2 (SHIP2), is a 5'-phosphatase that hydrolyses PIP3 into phosphatidylinositol (3,4)-biphosphate (PIP2) (Figure 3). SHIP2, a 142 kDa protein encoded by *INPPL1* gene, is majorly expressed in skeletal muscle, cardiac muscle and brain, and consists of an N terminal SH2 domain, a C terminal proline rich domain (PRD) and a catalytic 5'- phosphatase core domain (Figure 4)[61].

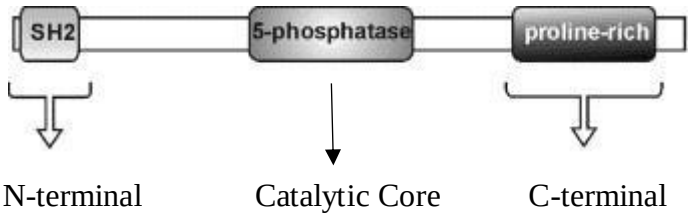


Figure 4. SHIP2: A structural illustration.

The protein consists of an N- terminal SH2 domain, a core catalytic 5'-phosphatase domain and the C-terminal proline-rich (PRD) domain[61].

It has histidine (His) and aspartic acid (Asp) active site pair essential for its enzymatic activity[60]. In 3T3-L1 cells, SHIP2 attenuates glucose uptake[62] whereas in L6 myoblasts, it inhibits phosphorylation of Akt and GSK-3β and thus negatively regulates insulin induced glycogen synthesis[63]. On the other hand, the expression of the dominant-negative (DN) forms of SHIP2 lacks its 5'-phosphatase activity to hydrolyze PIP3 in 3T3-L1 adipocytes and L6 myotubes and enhances insulin effects in glucose uptake and glycogen synthesis through its secondary messenger and intracellular effectors [62,63]. SHIP2 knock out enhances insulin stimulated Akt phosphorylation in mice model. However, glucose homeostasis and glucose tolerance remain normal[64]. Moreover, small molecule inhibitors of SHIP2 ameliorate the plasma glucose level in the rodent models of diabetes and insulin resistance[24].

Clement *et al* (2001) generated SHIP2 knockout ($SHIP2^{-/-}$) mice by deleting exons 19-29 of *INPPL1* gene including the active Asp site. The deletion also disrupted the second gene locus Phox2a. As a result, they reported rapid development of lethal hypoglycemia in the mice which died within three days of birth. Further, they noticed increased insulin sensitivity and glucose tolerance in the skeletal muscles of adult mice heterozygous for SHIP2 gene ($SHIP2^{+/-}$), which led to an increased plasma membrane translocation of GLUT4 transporter and elevated glycogen synthesis[65]. In the subsequent study of Sleeman *et al.* (2005), the more viable $SHIP2^{-/-}$ mice without the disruption of second locus, when fed on high fat diet, showed obesity resistance and increased insulin sensitivity[64]. Moreover, rats on high fat diet when treated with SHIP2 antisense oligonucleotides (SHIP2-AS) showed increased muscle insulin sensitivity and glucose tolerance indicating a critical role of SHIP2

in diet-induced obesity and insulin resistance[66]. The liver specific expression of DN SHIP2 in hyperglycemic-hyperinsulinemic KKA^y mice model increased hepatic glycogen synthesis and improved glucose tolerance[67]. Nakatsu *et al.* (2010) demonstrated that SHIP2 performs a role in regulating the endocytic clathrin-coated pit dynamics by dephosphorylating PIP2 in addition to PIP3 and thus defined a major site of its action which could be critical for understanding insulin signaling related dysregulation[68].

2.2.2.3 Single Nucleotide Polymorphisms (SNPs) of SHIP2

The human *INPPL1* gene is in the chromosome 11q13-14 and is associated with T2DM and metabolic syndromes such as hypertension and obesity. Marion *et al.* (2002) first reported the association of *INPPL1* gene mutation with T2DM in rats and humans. The authors showed that Goto-Kakizaki (GK) and spontaneously hypertensive rats (SHR) with R1142C mutation (substitution of an arginine to a cysteine at amino acid 1142) are genetically prone to T2DM and/or insulin resistance due to slight impairment of insulin signaling. As a result, they identified a heterozygotic deletion of 16bp sequence containing an ATTTA pentamer of an adenylate/uridylate-rich element in the 3'-untranslated region (UTR) in one of the eight diabetic subjects. This mutation enhanced the gene expression levels in vitro, indicating their importance in mRNA stability and translation efficiency. The researchers also determined the frequency of Δ16bp (16 bp deletion) mutated allele in 415 diabetic subjects from the United Kingdom and Belgium against 567 healthy controls. They found that 9 subjects carrying the mutant allele belonged to the diabetic cohort, versus 3 subjects in the control group. 7 patients out of 9 were hypertensive and 5 patients were obese[69].

In the subsequent study, the same group of researchers identified *INPPL1* SNPs associated with the metabolic syndrome. They resequenced the *INPPL1* gene of 15.2 Kb extensively, including all introns and exons, in a group of 64 people and further analyzed 11 markers and previously published 16bp deleted sequence. They genotyped the chosen markers in 1,304 participants from 424 British families with T2DM with confirmed Hardy- Weinberg equilibrium. In this Diabetes in family (DIF) study collection, hypertension was strongly associated with a group of 3 SNPs which were also strongly associated with central obesity. Subsequently, the researchers genotyped the same polymorphisms in 905 French T2DM patients and 305 control individuals. There was no association found between the genotype and haplotype frequencies and T2DM in the case-control study. Though, when the French diabetic patients with hypertension and without hypertension were compared, it showed strong link between the insertion (I) allele and hypertension. Moreover, the frequency of the most common haplotype was significantly higher in the French diabetic patients

with hypertension than those who had only hypertension but no diabetes[70]. However, another group of researchers could not confirm the significant association between essential hypertension (EH) and the 3 SNPs identified by Kaisaki *et al*. This outcome indicated that the role of SHIP2 variants could be specifically limited to hypertensive metabolic syndrome patients[71].

In 2005, Kagawa and colleagues identified 10 additional SNPs with a group of haplotypes (SNPs 1-3) and 4 missense mutations-containing SNPs (SNPs 3-6) in a cohort of 106 T2DM patients and 100 control individuals in a Japanese population. Their analysis revealed that the haplotype was more frequent in nondiabetic control than diabetic patients and one SNP, L632I (substitution of a leucine to isoleucine at amino acid 632) was located in the 5-phosphatase domain of the enzyme suggesting that the mutation might protect against insulin resistance. The protective role of SNP3-hSHIP2 (L632I) was confirmed further in vitro, in which insulin signaling was enhanced and the inhibition of PIP3 signal and Akt2 phosphorylation was reduced compared to WT-hSHIP2[72]. Another study in a Japanese cohort was carried out to identify SNPs on human *INPPL1* gene promoter and in 5'-UTR region[73]. The researchers found several SNPs in their study, among which 3 SNPs formed haplotypes and were associated with impaired fasting glycemia (IFG). Also, SNP-hSHIP2 which was present more frequently in IFG group compared to normal group showed increased promoter activity in vitro when inserted in luciferase reporter plasmid.

Hao *et al* investigated the association between the SNPs on *INPPL1* gene and T2DM pathogenesis in Chinese Han cohort. In their study, (+1893CC/AA) locus of *INPPL1* gene in T2DM individuals had significantly different genotype and allele frequency compared to that between the healthy control individuals. Moreover, G allele of (+2945A/G) locus was found to make the T2DM patients more susceptible to hypertension[74]. More recently in 2012, Hyvönen *et al* investigated the association of *INPPL1* gene SNPs with the metabolic syndrome and diabetic nephropathy in Finnish T1D patients. In their study, they identified two SNPs associated with the metabolic syndrome in men with T1D but not with diabetic nephropathy[75]. Interestingly, one of these SNPs was found to be associated with the metabolic syndrome in a British cohort analyzed earlier by Kaisaki *et al*[70]. These genetic studies demonstrate SHIP2 as a crucial therapeutic target of diabetes and warrant further research in this area.

2.3 Insulin sensitizing agents/small molecules

Oral anti-diabetic drugs can be classified into three categories: (1) Insulin sensitizing agents (ISA) (biguanide, thiazolidinediones), (2) Insulin secretagogues (glinide, sulfonylurea), (3) α1-glucosidase inhibitors[76]. Apart from oral drugs, incretin mimetics such as exenatide and amylin analogues such as

pramlintide are injectable anti-diabetic drugs. Effects of all the oral antidiabetic drugs except α1-glucosidase inhibitors depend on the enough level of insulin or pancreatic β cell function. The insulin secretagogues act primarily by stimulating impaired insulin secretion and thus depend on the functional β cells, whereas ISA complement the action of endogenous or exogenous insulin and enhance inulin sensitivity to reduce the blood glucose levels. Secretagogues are less recommended compared to sensitizers since their effective action depends on the optimal residual β-cell function. Moreover, sulfonylurea in combination with insulin often results into marginal failure to produce its effects and the metabolic improvements and glycemic control are short-lived and transient. Sulphonylureas combined with insulin and glinides do not reduce HbA1c significantly (1-2%) either. The use of α1-glucosidase inhibitors as antidiabetic drugs is limited due to their gastrointestinal side-effects such as stomachache, flatulence, diarrhea[76].

Besides the established antidiabetic drugs, targeting disordered insulin signaling pathway is the new approach in diabetic research. One such approach is to discover novel small molecule inhibitors of SHIP2 enzyme which negatively regulates insulin signaling pathway[64]. For instance, Suwa *et al* first time reported the discovery of novel small molecule inhibitor of SHIP2, AS1949490[24].

2.3.1 Biguanides

For the management of T2DM, the first-line prescribed anti-diabetic drug of choice is a biguanide, metformin (1,1-dimethylbiguanide) (Figure 5)[77,78]. The extended-release tablet dosage in the initial stages is 500 mg per day, usually with supper. The recommended dosage increment is up to 2000 mg[79]. Apart from its use in the management of T2DM, metformin has therapeutic applications in other disease conditions such as cardiovascular diseases (CVD), DN, polycystic ovary disease and cancer[80]. It is an antihyperglycemic agent without the risk of overt hypoglycemia found in the case of secretagogues such as sulfonylureas.

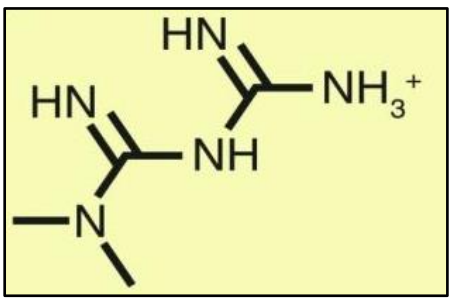


Figure 5. Chemical structure of metformin: two coupled guanidine molecules containing additional substitutions[78]

It has been shown that at millimolar concentrations, metformin mediates the suppression of hepatic gluconeogenesis and an increase in peripheral glucose uptake by inhibiting mitochondrial respiratory chain complex 1[81,82], by activating the cellular energy sensor AMPK (AMP activated protein kinase)[83] or by mechanisms independent of AMPK[84]. Madiraju *et al.* (2014) identified mitochondrial glycerophosphate dehydrogenase (mGPD) as an important molecular target of metformin in the liver. Suppression of mGPD by metformin leads to the inhibition of hepatic gluconeogenesis[82]. Recently the same authors revealed that at clinically relevant plasma and liver concentrations of metformin, it suppresses hepatic gluconeogenesis via its inhibitory effects on mGPD in a redox-dependent manner *in vivo*. This revelation indicated the less significance of the previously described metformin mechanism of action involving mitochondrial complex I, AMPK-mediated ACC inhibition or reduced ectopic lipid accumulation in liver in suppressing hepatic gluconeogenesis[85]. However, the authors did not describe the mechanism of action of metformin and its direct target in peripheral tissues.

Recently, the Lehtonen lab, where this thesis was carried out, revealed for the first time the mechanism of action of metformin to increase glucose uptake in peripheral tissues[86]. Using cultured myotubes and db/db mice models, the group has shown that metformin directly targets SHIP2's catalytic phosphatase domain and reduces its activity, but not expression, in cultured myotubes and skeletal muscle cells and podocytes of diabetic mice, thereby increases insulin sensitivity and glucose uptake in peripheral tissues. However, they revealed that metformin does not reduce gluconeogenesis in the liver by suppressing SHIP2 activity, the reasons for which are unclear. Moreover, metformin increases GLUT4 translocation at plasma membrane[87] and slows down its endocytosis[86]. In the kidneys of patients with T2DM, metformin suppresses the enhanced activity of SHIP2. In podocytes too, it normalizes the reduced-Akt activity induced by SHIP2 overexpression, thereby acting renoprotectively[86]. Thus, in the liver, metformin acts in a way to reduce gluconeogenesis[82, 85], whereas in peripheral tissues, it enhances glucose uptake through suppression of SHIP2 activity[86].

2.3.2 Thiazolidinediones (TZDs)

TZDs are prescribed as ISA to the diabetic patients who are not advised to take metformin as a first line anti-diabetic drug[88]. TZDs are the ligands for peroxisome proliferator-activated receptors (PPARs), the transcription factors that upon stimulation by TZDs modify the transcription of genes involved in the metabolic pathways of glucose and lipid homeostasis and thus enhance insulin sensitivity in muscle cells, hepatocytes and adipocytes[89]. TZDs cause significant reduction of HbA1c

(0.5 % to 1.5%). However, these drugs cause weight gain, peripheral edema and cardiac failure due to associated risks of fluid retention. Initially, troglitazone, rosiglitazone and pioglitazone were the three TZDs that FDA approved. However, later, troglitazone was withdrawn from the market due the associated risk of liver failure. Moreover, meta-analysis studies showed the increased risk of cardiovascular events associated with rosiglitazone causing its withdrawal from the market in 2010. Later, in 2013, after further research, the restrictions were eased by FDA. Pioglitazone is known to decrease the risk of stroke. Though, there is a controversy about pioglitazone's association with increased risk of bladder cancer which needs further studies to clarify[90]. TZDs are associated with increased risk of bone fractures especially in women and elderly men[88].

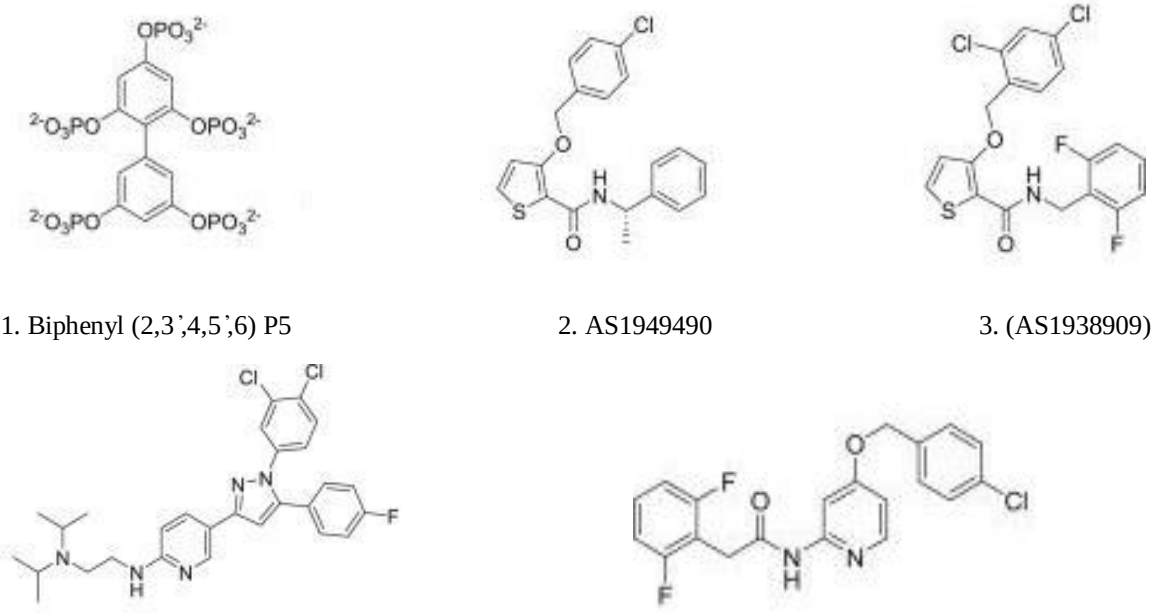
2.3.3 Small molecule inhibitors of SHIP2

PIP3 is an important secondary messenger of PI3K-Akt mediated insulin signaling pathway, and hydrolyzed form of this messenger by SHIP2 shifts the signaling pathway to PI(3,4)-dependent pathway. This indicates that SHIP2 is involved in impaired-insulin signaling pathway leading to several diseases including T2DM, and inhibiting its 5' phosphatase activity by small molecule inhibitors might be a significant therapeutic strategy[91]. The synthesis of many small molecule inhibitors of SHIP2 has been reported including phosphorylated biphenyl 2,3',4,5',6 pentakisphosphate (biphenyl(2,3',4,5',6)P5)[25] **(1)**, thiophene carboxamide compounds (AS1949490)[24] **(2)** and (AS1938909)[26] **(3)** by Astellas Pharma Inc. (Ibaraki, Japan), a pyrazole based drug NGD-61338[27] **(4)** and a pyridin based inhibitor *N*-[4-(4-chlorobenzyloxy)pyridin-2-yl]-2-(2,6-difluorophenyl)-acetamide (CPDA)[28] **(5)** (Figure 6)[91].

Vandeput and colleagues synthesized a series of benzene and biphenyl polyphosphates with more rigid phosphate regiochemistry than natural inositol phosphate and with different potency of inhibiting inositol 5 phosphatases. In this series, phosphorylated biphenyl compound, biphenyl (2,3',4,5',6) P5 was found to be the most potent inhibitor of SHIP2 with IC_{50}= 1.8µM. Moreover, this inhibitor was not SHIP2 substrate or SHIP2 specific since it was also found to inhibit another phosphatase, type I inositol 1,4,5- triphosphate 5-phosphatase. Also, the authors did not discuss the selective inhibition of SHIP1 or PTEN or the structures of the inhibitors in their study[25].

Using high-through put screening, Suwa and colleagues identified highly selective, potent and competent SHIP2 inhibitors, thiophene carboxamide compounds (AS1949490) and (AS1938909) (Figure 6) with IC_{50} values 0.62 µM and 0.57 µM respectively[24,26]. In L6 myotubes, both inhibitors enhanced Akt phosphorylation, glucose consumption and glucose uptake. These

inhibitors were reported to enhance glucose metabolism in L6 myotubes by significantly inducing the mRNA expression of GLUT1[24,26]. However, the analogues of these thiophene lead molecules with improved solubility, pharmacokinetic properties and cell permeabilities would need to be prepared as thiophenes have limited cell permeability and poor pharmacokinetic properties[92].



1. Biphenyl (2,3',4,5',6) P5 2. AS1949490 3. (AS1938909)

4. NGD-61338 5. CPDA

Figure 6. SHIP2 inhibitors: chemical structures[91]

Using Automated Ligand Identification System (ALIS), Annis *et al.,* (2009) identified a pyrazole based SHIP2 inhibitor, NGD-61338 with IC_{50}=1.1µM, one of the most potent compounds in their combinatorial libraries that were screened. The inhibitor binds to the same site as its natural substrate, PIP3[27]. However, the researchers did not discuss its selectivity with respect to other phosphatases, its effects in cells, bioavailability or *in vivo* toxicity studies.

Having no access to the crystal structure of SHIP2 catalytic domain at the time, Ichihara *et al.* released the data of a novel pyridine-based compound, CPDA using a ligand-based drug discovery (LBDD) strategy involving previously published NGD-61338 and AS1949490 as templates. Through in vitro assay, they could identify its greater potency to phosphorylate and enhance insulin signaling than AS1949490. In diabetic mice model, CPDA was found to ameliorate the impaired glucose metabolism significantly[28].

2.3.4 Need for novel small molecule SHIP2 inhibitors

Metformin is the first-line prescribed drug for T2DM in the absence of contraindications[93]. However, it is contraindicated in patients with chronic kidney disease (CKD), congestive heart failure and liver failure. Major side effects of metformin are gastrointestinal which are persistent in ~8% patients, thus the medication is discontinued. In patients with severe renal insufficiency, metformin may lead to lactic acidosis[94.95]. Especially in the case of patients with kidney injury and CKD, regulatory agencies have restricted its use. A severe metformin-associated lactic acidosis (MALA) could have lethal consequences due to multiple end-organ damage[93]. Moreover, metformin may interfere with B12 absorption leading to vitamin B12 deficiency besides the deficiency of folic acid[94,95].

The second-line agents of T2DM have their own contraindications and adverse effects. Hypoglycemia and weight gain are the primary side effects of sulfonylureas (SUs). Non-sulfonylurea secretagogues such as the class of medications known as glinides too may lead to hypoglycemia in patients with severe renal damages. TZDs are contraindicated in patients with cardiac failure and warrant extra caution in patients with peripheral vascular disease. Furthermore, α-glucosidase inhibitors have contraindications for gastrointestinal disorders besides liver cirrhosis and renal dysfunction. Amylin analog – pramlintide, an injectable drug, has contraindications in patients with gastroparesis (GP). It has also other gastrointestinal side effects.

Among newer T2DM medications than the described above, Dipeptidyl peptidase -4 (DPP-4) inhibitors are associated with increased risk of angioedema, upper respiratory tract infection (~3.0 – 9.0 % with sitagliptin) and urinary tract infection (~2.0 -5.0 % with sitagliptin)[96,97,98]. Glucagon like peptide -1 receptor agonists (GLP-1 RA) are not prescribed during pregnancy or lactation. The GLP-1 RA, Exenatide, is not advised to be prescribed to patients with severe renal damage[95]. Furthermore, sodium glucose co-transporters 2 inhibitors (SGLT2) are contraindicated in severe renal diseases. Urinary tract infections are also associated with this class of drugs. Insulin injections may precipitate cardiac failure[94]. Thus, the T2DM drugs that are already being prescribed have some common contraindications such as renal impairment and/or gastrointestinal problems.

Moreover, we still lack SHIP2 inhibitors that have applicable bioavailability, solubility and pharmacokinetics properties. Since the first-line prescribed drug of diabetes, metformin, enhances glucose uptake in peripheral tissues by reducing the 5' phosphatase activity of SHIP2 in cell cultures and in mice models[86], more potent inhibitors targeting the activity of SHIP2 could be designed. Especially, the SHIP2 inhibitors that could be applicable in T2DM patients with impaired

kidney function are of great interest so that the patients with renal injury or impaired kidney function could benefit from the drugs.

Hence, searching for novel SHIP2 inhibitors that are specific to SHIP2, have better solubility, pharmacokinetic and bioavailability properties with little to no contraindications could be a keystone strategy in diabetes research.

3 AIMS OF THE STUDY

Increasing number of patients with T2DM has necessitated the discovery of novel drug targets of T2DM due to lack of current optimal therapeutic options. Since insulin resistance is strongly associated with T2DM[1] and impaired insulin signaling pathway is one of the most important factors behind its causes[24], research in this direction could be a key strategy. SHIP2, a 5'phosphatase, is known to negatively regulate PI3K-Akt mediated insulin signaling pathway by hydrolyzing PIP3 to PIP2[62]. Moreover, overexpression of SHIP2 is associated with insulin resistance[66]. Hence, inhibition of 5' phosphatase activity of SHIP2 could be an important therapeutic strategy. In the last decade, several publications have reported identification of novel SHIP2 inhibitors [24,25,26,27,28]. However, they still lack drug like properties. In this research scenario,

the specific aims of the current thesis are:

1) To identify novel small molecule SHIP2 inhibitors by testing their ability to activate the insulin signaling cascade in murine L6 myotubes by measuring Akt activation as an indirect measure of SHIP2 inhibition
2) To test cytotoxicity of selected novel top candidate SHIP2 inhibitors in L6 myotubes

4 MATERIALS AND METHODS

4.1.1 Cell Culture

4.1.1.1 Maintenance of L6 Rat myoblasts

L6 Rat myoblasts (CRL-1458™; ATCC®, Manassa, VA) were maintained as described earlier[99] in high-glucose DMEM medium supplemented with 10% Fetal Bovine Serum (FBS, Sigma, St. Louis, MO, USA), 1% Ultraglutamine (Lonza, Basel, Switzerland) and 1% penicillin/streptomycin (P/S) (Sigma) in 5% CO_2 at +37°C. The medium was changed every 48 hours except on weekends when the medium was changed early morning on Mondays.

4.1.1.2 Splitting 10 cm continuation plate and seeding 12 well and/or 96 well plates

The myoblasts were washed with ~10 ml sterilized PBS and then detached from the surface of dish by adding ~2 ml trypsin. The cells were incubated for around 4 mins at 37°C in the incubator followed by addition of ~7 ml fresh proliferation medium to trypsin containing cells once the cells were detached and free flowing in the medium. The cells were mixed well in falcon tube and centrifuged at 900 rpm for 4 mins. The supernatant was discarded and 4 – 5 ml fresh medium was added to the pellets. To count the cells, 10 µl cells were taken on each side of the Bürker chamber. For 10 cm continuation plate, ~20×10^4 cells on weekdays or ~18×10^4 cells on weekends were seeded in 10 ml fresh medium. For 12 well plates, 2.0×10^4 cells/ml/well or 1.8×10^4 cells/ml/well were seeded whereas for 96 well plates, 0.2×10^4 cells/100µl/well or 0.18×10^4 cells/100 µl/well were seeded depending on weekdays or weekends. Following formula was used to calculate the volume required to seed the respective number of cells.

Required volume of cells V = (required number of cells / concentration of cells), where concentration of cells is the number of cells counted in Bürker chamber

The cells were split until they reached passage 8.

4.1.1.3 Differentiation into myotubes

Differentiation of myoblasts into myotubes for experiments in 12 well or 96 well plates was initiated by feeding the myoblasts a fresh differentiation medium (high glucose DMEM supplemented with 2% FBS, 1% ultraglutamine and 1% P/S) once they reached 60-70% confluence in 12 or 96 well plates. The differentiation of L6 myoblasts into myotubes takes around 7-10 days depending on the passage number of the myoblasts. Fresh differentiation medium was fed every other day.

4.1.1.4 Treatment of myotubes with inhibitors

4.1.1.4.1 Starvation of myotubes

Once the myoblasts were completely differentiated into myotubes in 12 well plates after 7-10 days of differentiation period, the myotubes were starved at 37°C in starvation medium (SF medium) for 16-20 hours. To treat the myotubes with inhibitors in the morning, the starvation was initiated in the previous evening. The old differentiation medium was aspirated and 1 ml fresh SF medium/well was added and the plate was agitated well before incubating for 16-20 hours at 37°C.

4.1.1.4.2 Inhibitor preparation and treatment

The novel small molecule inhibitors were prepared in DMSO by the collaborator lab and the stock concentration was 100mM. Before the treatment, they were diluted to 1, 10 and 25 µM concentrations in SF medium. For control, the same volume of DMSO as the volume of inhibitor to make 25 µM was diluted to fresh SF medium. After 16-20 hours of starvation period, the old medium was removed and 1 ml SF medium/well containing the inhibitors was added followed by incubation for 15 mins at ~37°C.

4.1.1.4.3 Insulin treatment

After 15 mins of incubation with inhibitors, 10 µl of 1 µM Actrapid® human insulin (stock solution, Novo Nordisk Limited, Denmark) was added to each well (final concentration 10 nM) containing SF medium + control or inhibitors of three different concentrations and further incubated for 15 mins at 37 °C. The experimental scheme of the 12 well plate is following (Figure 7):

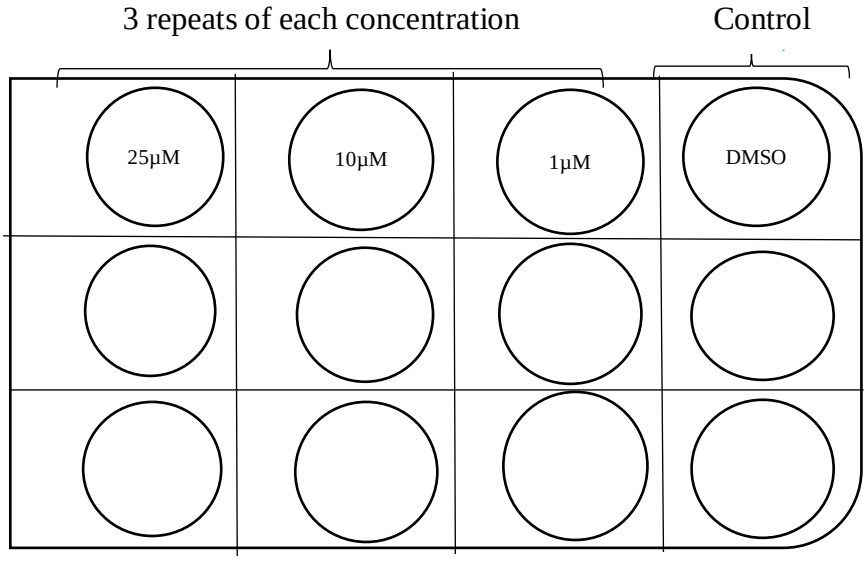


Figure 7. Experimental scheme of the 12 well plate: inhibitor treatment

4.2.2 Cell Lysis

Following insulin stimulation for 15 mins, the myotube plates were immediately kept on ice and the cells were washed with ice-cold Phosphate-buffered saline (PBS) for three times while keeping the plates on ice box. It was followed by scrapping the cells into ice-cold lysis buffer [1% Nonidet P-40 (NP40) purchased from BDH, 150mM NaCl salt, Tris-HCl, pH 7.4, 50mM NaF, 1× protease inhibitor cocktail (Roche (#04693116001), 1mM Na_3VO_4] and transferred to Eppendorf tubes to rotate for 15 mins at 4 ℃. Following rotation, the cells were centrifuged at 16,000 g at 4 ℃ for 10 mins and the supernatant was collected to measure the protein concentration.

4.2.3 Bradford assay and sample preparation

To prepare the samples for immunoblotting, protein concentration was measured using Bradford method. For this, 800 µl milli-Q water, 5 µl sample and 200 µl Bradford reagents (Bio-Rad) were mixed well in 1 ml plastic cuvettes. For reference reading, 5 µl of lysis buffer was mixed instead of cell lysate. The OD595 values of the samples were read after 5 mins of incubation on bench and protein concentrations in samples were calculated using a standard curve. Using the protein concentration, volume for 20 µg of protein was calculated and diluted with 4 × Laemmli Sample Buffer containing 10% β-mercaptoethanol. The samples were boiled at 100 °C for 10 mins and run on SDS-PAGE straight away or incubated at 100 °C for 5 mins and frozen at -20 °C to run later.

4.2.4 Immunoblotting

Protein samples were separated by 8% SDS-PAGE and transferred onto PVDF-FL membranes (Millipore, Billerica, MA) prewetted in MeOH (methyl alcohol) for 1-2 mins and equilibrated with 1 × transfer buffer [100 ml 10× transfer buffer (19.8mM glycine + 153mM Tris), 200 ml MeOH, 700 ml milli-Q water)] for ~2 mins, followed by a sandwich assembly of fiber pads, filter papers, gels and membranes as shown in figure 8. All the components were well soaked with transfer buffer and locked into a transfer cassette as a sandwich. Special care was taken to avoid air bubbles between the components of the transfer sandwich. The transfer cassette was immersed in transfer apparatus filled with transfer buffer. The transfer apparatus was kept in a box containing ice blocks and the transfer of protein samples was carried out at constant 70 V for 2 hours (or at constant 45-55 mAmp for 16-18 hours or at constant 60 mAmp for 12 hours).

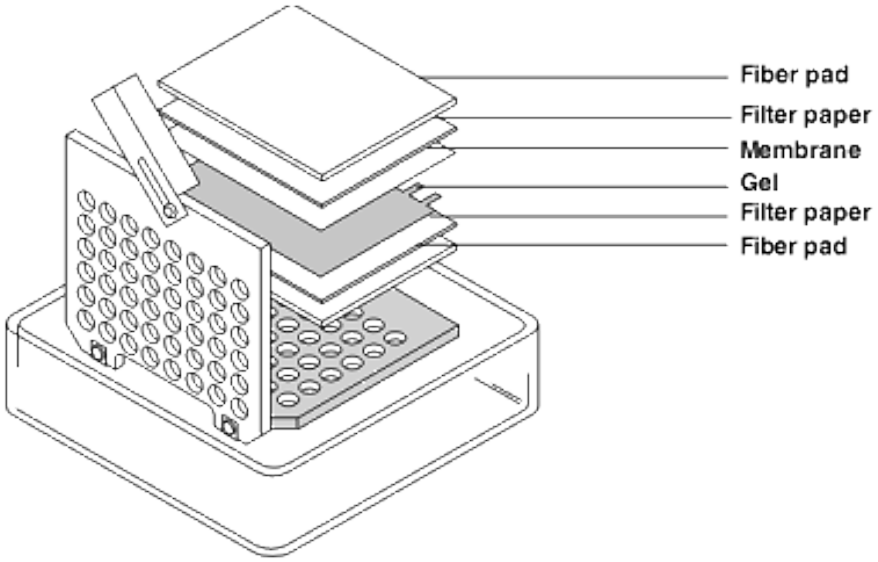


Figure 8. Transfer of protein samples from SDS-GEL to PVDF membrane: a sandwich assembly of the components of transfer cassette (Laboratory protocol)

4.2.4.3 Immunodetection:

Once the transfer was over, the membrane was rinsed with milli-Q water and stained with Ponceau-S followed by the final rinse with milli-Q water to detect the protein bands. The membranes were cut with razor blade to get the required parts of the membranes containing the proteins of interest for further treatments and the rest of the membranes were discarded. The membranes were blocked in Odyssey blocking buffer (OBB) (LI-COR, Lincoln, NE) (diluted 1:1 with Tris Buffered Saline-TBS) for 1 hour at room temperature. Following blocking, the membranes were incubated with primary antibodies rabbit anti-phospho-Akt (Ser473) (#9271, Cell Signalling Technology, Danvers, MA) and mouse anti-Pan Akt (MAB2055, R&D systems, Minneapolis, MN) (1:1000 in OBB, diluted 1:1 with 0.2% tween 20 in TBS) overnight at 4 °C. For housekeeping genes, the membranes containing the bands around 40-50 kDa were incubated with primary antibodies mouse anti-actin (Sigma, A3853) or mouse anti-α-tubulin (Sigma, T6199) (1:2000 or 1:5000 in OBB respectively). The following day, membranes were rinsed with TBS + 0.1% Tween 20 and washed for 15 mins three times with TBS +0.1 % Tween 20. After getting rid of unbound primary antibodies, the membranes were incubated with secondary antibodies Alexa Fluor 680 (#A10038, Invitrogen) donkey anti-mouse and/or IR800 (#926-32213, LI-COR) donkey anti-rabbit IgGs (1:10000 in OBB, diluted 1:1 with 0.2% tween 20 in TBS + 0.01% SDS respectively). Detection and quantitation with an Odyssey Infrared Imager were done following the rinsing and washing the membranes thrice with TBS + 0.1% tween 20 for 15 mins.

4.2.5 Cytotoxicity test

For alamarBlue® cytotoxicity test, 0.2×10^4 or 0.18×10^4 myoblasts/ well were seeded in 96 well plates using multichannel automatic pipette and differentiated into myotubes after 48 hours, which took around 7-10 days for myoblasts to differentiate into myotubes as described in 4.2.1.

4.2.5.1 Preparation of inhibitor dilutions:

Four different concentrations [50, 100, 200, 300 (µM)] of selected top candidate inhibitors were prepared as following:

C_{300} – 4.5 µl in 1.5 ml differentiation medium

C_{200} – 1 ml of C_{300} in 500 µl differentiation medium

C_{100} – 750 µl of C_{200} in 750 µl differentiation medium

C_{50} – 750 µl of C_{100} in 750 µl differentiation medium

4.2.5.2 Treatment with inhibitors and assessment of cell viability using alamarBlue® reagent:

As shown in figure 9, 100 µl/well of four different concentrations were added in parallel wells for each selected inhibitor, each having four repeats. The myotubes were incubated with inhibitors at 37 °C for 20 hours. Following incubation, 10 µl of alamarBlue® reagent was added in each well and fluorescence intensity (excitation wavelength 560nm, emission wavelength 590 nm) was immediately measured using the Hidex Sense application, followed by the second measurement at the interval of 45 mins and the third measurement at the interval of 60 mins. Between the measurements, the myotubes were incubated at 37°C. The percentage of living cells was derived by comparing it to the control cell.

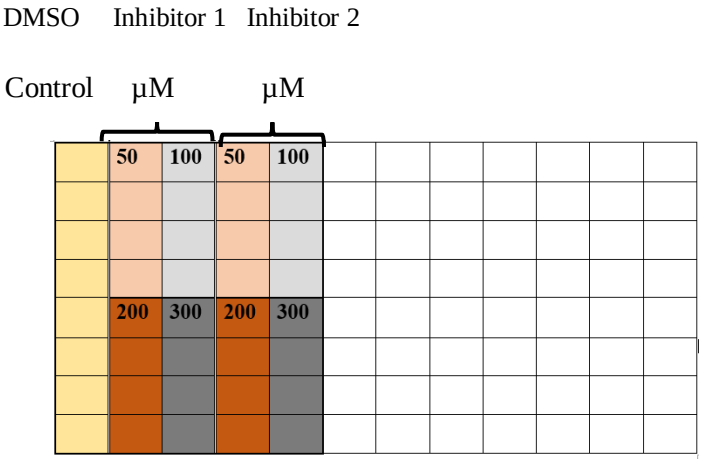


Figure 9 Experimental scheme of 96 well plate for alamarBlue® cytotoxicity assay

5 RESULTS

5.1 Treatment of L6 myotubes with SHIP2 inhibitors activates Akt

Inhibition of 5'- phosphatase activity of SHIP2 enzyme increases the phosphorylation of Akt and its activity[100]. In the present study, 8 novel small molecule SHIP2 inhibitors, #160, #161, #162, #163, #167B, #170A, #171, #172 (due to the fact that the inhibitors are not yet secured under intellectual property rights, the chemical structures of the inhibitors have not been revealed in this study) were tested for their capacity to phosphorylate Akt to pick up the best candidate using the lysates of L6 myotubes as described in materials and methods. These molecules were modified from the single candidate from the original hits found in the structure-based virtual screening[86].

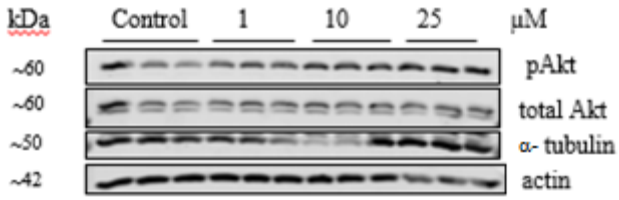


Figure 10. Immunoblot detection of insulin-induced Akt phosphorylation in protein lysates of L6 myotubes using antibodies that detect phosphorylated Akt and total Akt. Actin and α-tubulin were used for normalization.

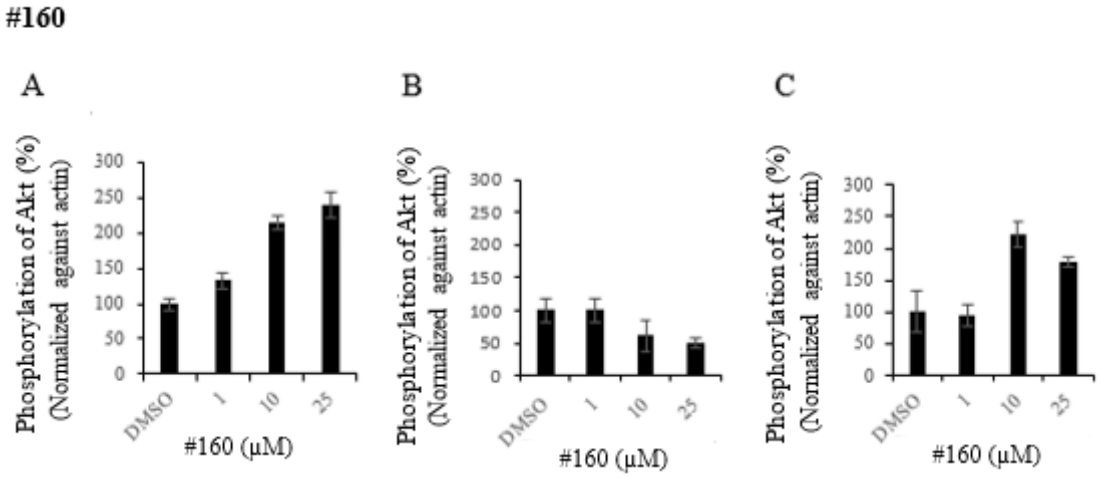


Figure 11. Effect of #160 on insulin-stimulated Akt phosphorylation in L6 myotubes. The level of Akt phosphorylation in myotubes treated with DMSO (vehicle) was set to 100% (control). A, B, C indicate the graphs representing the three independent experimental trials each containing three repeats. The L6 myotube lysates were immunoblotted (not shown) against p-Akt or pan-Akt antibodies and the signal output was converted to percent activation by normalizing against house-keeping gene actin. The values are the means ± relative standard deviation of three experimental repeats in each trial

Using immunoblotting, insulin induced Akt phosphorylation on Ser473 was detected by phospho-Akt (p-Akt) antibody at molecular weight ~60 kDa and was quantified by normalizing against housekeeping genes actin or α-tubulin (Figure 10)[24].

Graphs in figure 11 (A, B, C) show the three independent trials of inhibitor #160 treatment with L6 myotubes. In the first trial (A), the Akt phosphorylation levels in the cells treated with 10 µM or 25µM of #160 were greater than 2-fold than in those treated with DMSO (control) (Figure 11, A). However, the same results could not be repeated in the subsequent trials (Figure 11, B, C).

#161

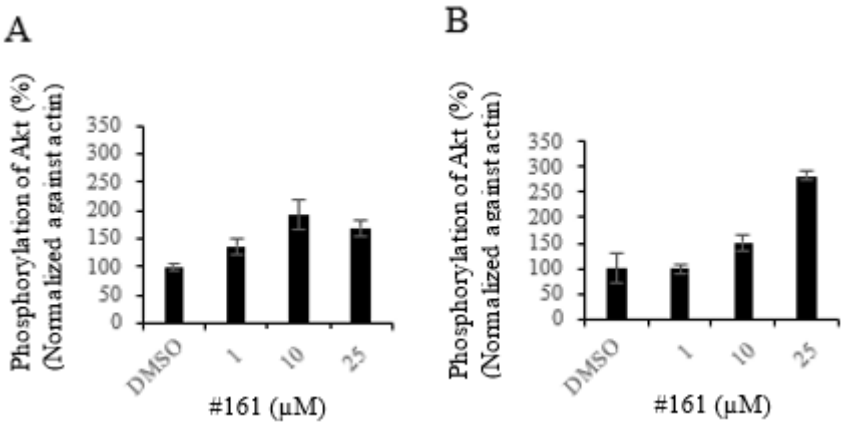


Figure 12 Effect of #161 on insulin-stimulated Akt phosphorylation in L6 myotubes. The level of Akt phosphorylation in myotubes treated with DMSO (vehicle) was set to 100% (control). A and B indicate the graphs representing the two independent experimental trials each containing three repeats. The L6 myotube lysates were immunoblotted (not shown) against p-Akt or pan-Akt antibodies and the signal output was converted to percent activation by normalizing against house-keeping gene actin. The values are the means ± relative standard deviation of three experimental repeats in each trial

In trial A of the treatment with the inhibitor #161, the phosphorylation levels of Akt were greater (~1.9-fold greater than control) at 10µM concentration of the inhibitor than at 1 µM (~1.3-fold greater than control) (Figure 12, A). However, at 25 µM concentration, the phosphorylation levels dropped down to ~1.6-fold compared to the control. In the subsequent trial (Figure 12, B), the Akt phosphorylation was ~3-fold greater than in the control. In two trials (Figure 12, A, B), the phosphorylation levels of Akt varied at different concentrations of the inhibitor which made it difficult to select the inhibitor for further analysis.

The Akt phosphorylation levels were ~1.5-fold greater than in the control or less than that in the three different experimental trials when treated with three different concentrations of #162 (Figure 13, A, B, C).

#162

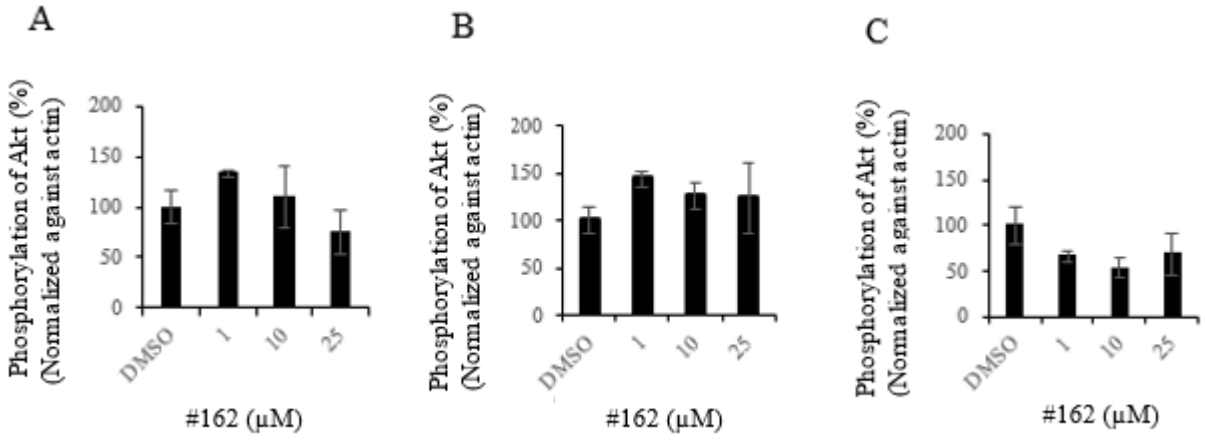


Figure 13. Effect of #162 on insulin-stimulated Akt phosphorylation in L6 myotubes. The level of Akt phosphorylation in myotubes treated with DMSO (vehicle) was set to 100% (control). A, B, C indicate the graphs representing the three independent experimental trials each containing three repeats. The L6 myotube lysates were immunoblotted (not shown) against p-Akt or pan-Akt antibodies and the signal output was converted to percent activation by normalizing against house-keeping gene actin. The values are the means ± relative standard deviation of three experimental repeats in each trial

#163

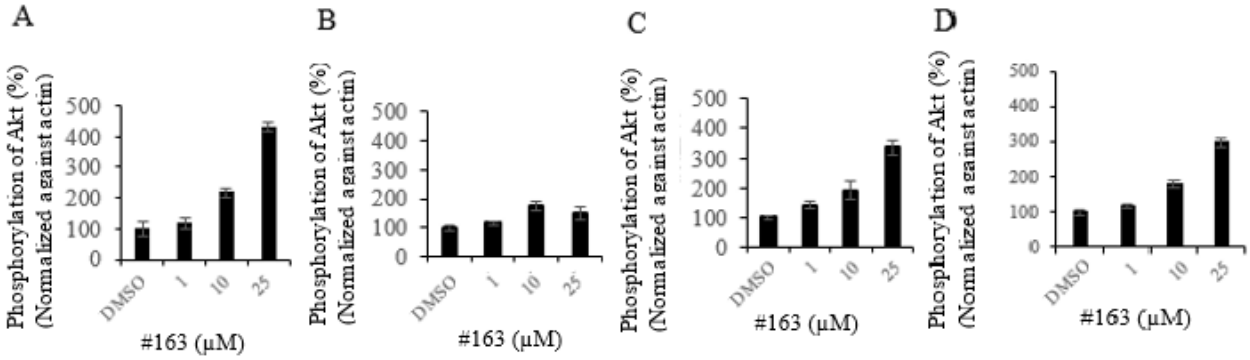


Figure 14. Effect of #163 on insulin-stimulated Akt phosphorylation in L6 myotubes. The level of Akt phosphorylation in myotubes treated with DMSO (vehicle) was set to 100% (control). A, B, C and D indicate the graphs representing the four independent experimental trials each containing three repeats. The L6 myotube lysates were immunoblotted (not shown) against p-Akt or pan-Akt antibodies and the signal output was converted to percent activation by normalizing against house-keeping gene actin. The values are the means ± relative standard deviation of three experimental repeats in each trial

Inhibitor #163 increased the phosphorylation of Akt in a concentration dependent manner. The phosphorylation levels were ~3-fold or greater than 3-fold at 25 µM concentration of the inhibitor compared to the control in trials A, C and D when normalized against actin (Figure 14, A, C, D). The result could not be repeated in one of the four trials (Figure 14, B) where increase in Akt phosphorylation was less than 2-fold compared to the control. Though, the inhibitor #163 was selected for cytotoxicity test as the results were consistent in three trials (Figure 14, A, C, D).

The results of the trials with inhibitor #167B were highly inconsistent and could not be repeated (Figure 15).

#167B

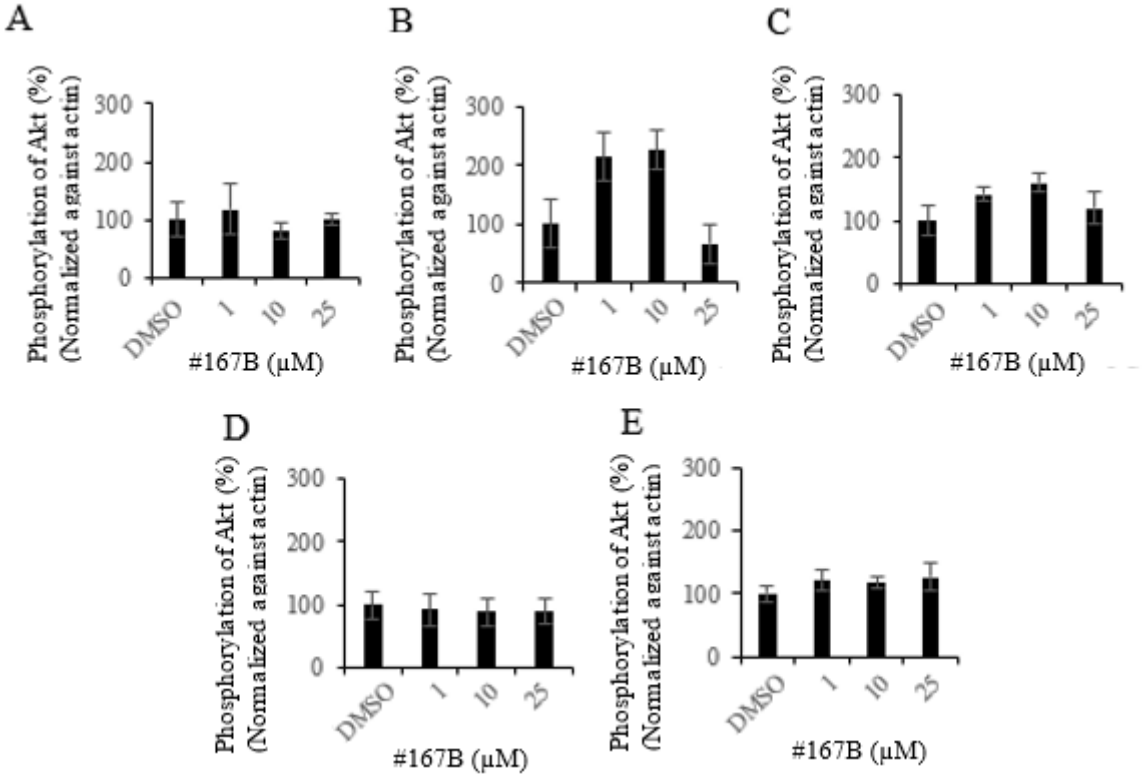


Figure 15. Effect of #167B on insulin-stimulated Akt phosphorylation in L6 myotubes. The level of Akt phosphorylation in myotubes treated with DMSO (vehicle) was set to 100% (control). A, B, C, D and E indicate the graphs representing the five independent experimental trials each containing three repeats. The L6 myotube lysates were immunoblotted (not shown) against p-Akt or pan-Akt antibodies and the signal output was converted to percent activation by normalizing against house-keeping gene actin. The values are the means ± relative standard deviation of three experimental repeats in each trial

Treatment with Inhibitor #170A increased the phosphorylation of Akt in a concentration dependent manner in trial A (Figure 16, A) which was consistent with the treatment with #163 (Figure 14, A, C, D). However, the trials could not be repeated (Figure 16, B, C). Inhibitor #170A was chosen nevertheless along with inhibitor #163 to assess the cytotoxicity test after discussing the results with the other members of the lab working on the same project.

#170A

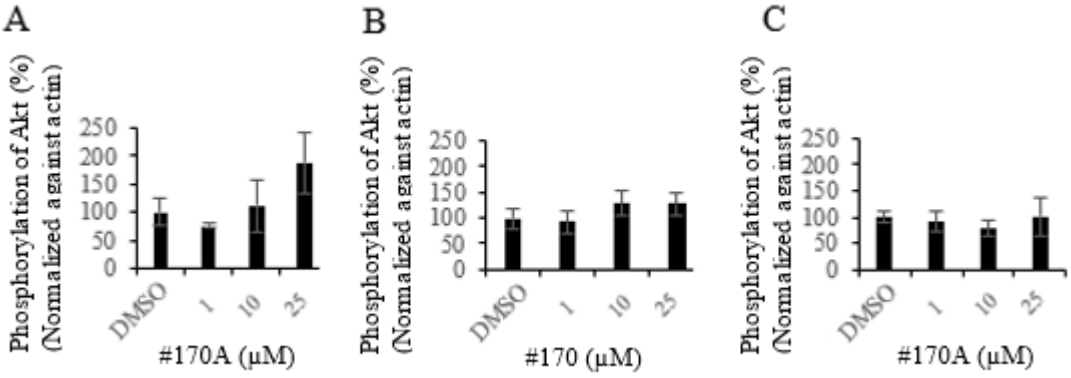


Figure 16. Effect of #170A on insulin-stimulated Akt phosphorylation in L6 myotubes. The level of Akt phosphorylation in myotubes treated with DMSO (vehicle) was set to 100% (control). A, B, C indicate the graphs representing the three independent experimental trials each containing three repeats. The L6 myotube lysates were immunoblotted (not shown) against p-Akt or pan-Akt antibodies and the signal output was converted to percent activation by normalizing against house-keeping gene actin. The values are the means ± relative standard deviation of three experimental repeats in each trial

In order to investigate the stability of the house-keeping gene expression under the current experimental conditions in L6 myotubes to get reliable results, I decided to normalize the test results of #171 and #172 with actin as well as α-tubulin. The graphs in figure 17 (A) shows ~ 1.75-fold increase in the Akt phosphorylation level compared to control at 10 μM concentration of #171 when the values were normalized against both actin and α-tubulin house keeping genes separately in the same blot. The graph patterns looked similar when normalized against two different house keeping genes. The inhibitor showed reduced Akt phosphorylation at 25 μM compared to the control when normalized against actin, whereas, it slightly enhanced the Akt phosphorylation at the same concentration compared to the control when normalized against α-tubulin (Figure 17, A). However, in the subsequent trial, Akt phosphorylation was enhanced at 1 μM concentration whereas at higher concentrations, it was lower than the control when normalized against actin or α-tubulin (Figure 17, B). Graphs in figure 17 (C) show ~4.5-fold increase in Akt phosphorylation at 10 μM concentration and ~3.5-fold increase at 25 μM concentration when normalized against α-tubulin whereas when actin

was used for normalization, no differences were observed. In the fourth trial (D), the phosphorylation level of Akt increased ~1.25 fold only at 25 µM when the signal was normalized against actin.

#171

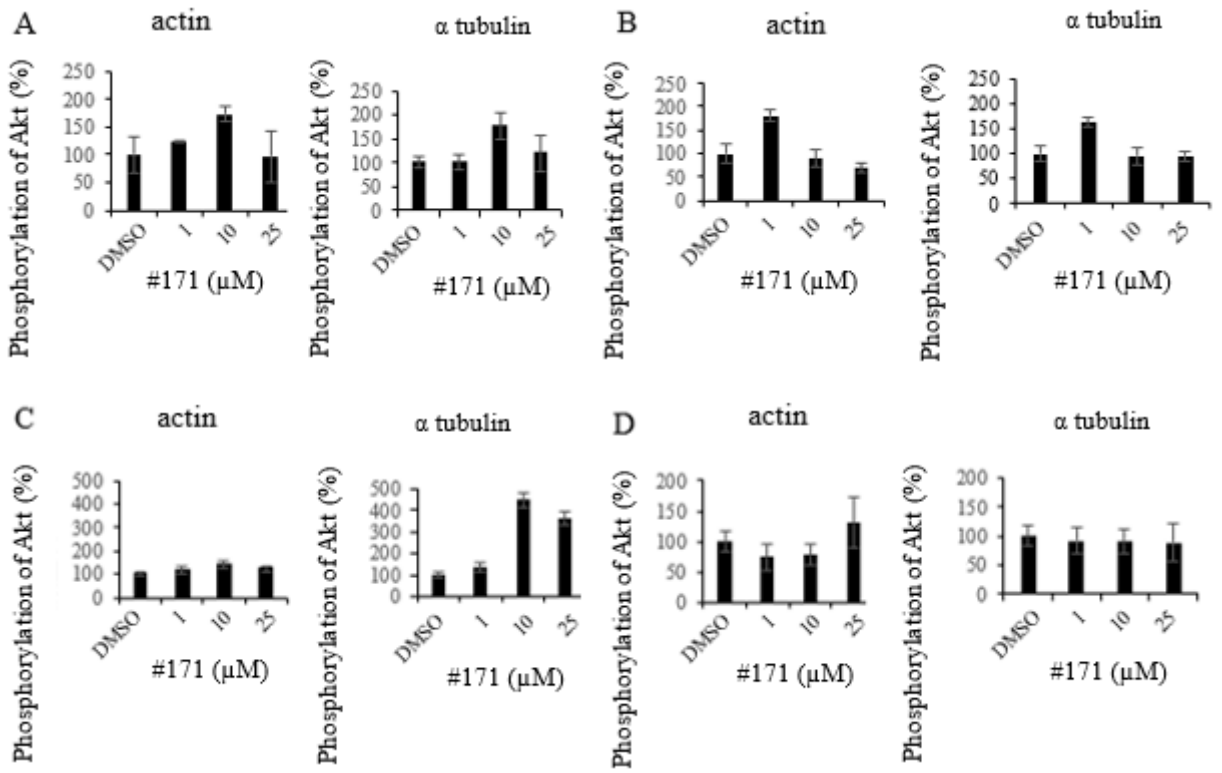


Figure 17. Effect of #171 on insulin-stimulated Akt phosphorylation in L6 myotubes. The level of Akt phosphorylation in myotubes treated with DMSO (vehicle) was set to 100% (control). A, B, C and D indicate the graphs representing the four independent experimental trials each containing three repeats. The L6 myotube lysates were immunoblotted (not shown) against p-Akt or pan-Akt antibodies and the signal output was converted to percent activation by normalizing against house-keeping gene actin or α-tubulin. The values are the means ± relative standard deviation of three experimental repeats in each trial

Akt phosphorylation level in myotubes treated with #172 was around control level or below control level when normalized against actin or α-tubulin (Figure 18, A). In the subsequent trial (B), the Akt activity was ~1.25-fold greater than in the control at 10µM when normalized against actin. In the same trial, at 25 µM, Akt activity was reduced compared to the control. However, normalization against α-tubulin showed that the phosphorylation level of Akt was lower than in the control at all concentrations. Interestingly, in the third trial, Akt phosphorylation was enhanced the most at 25µM concentration whereas none of the concentrations of the inhibitor enhanced the phosphorylation of Akt above the control when normalized against α-tubulin. It is noteworthy that in

the fourth attempt, the Akt phosphorylation level was almost 1.5-fold greater than in the control at 1 µM when normalized against actin and almost 2.0-fold greater when normalized against α-tubulin at the same concentration. In comparison to 1µM concentration, higher concentrations in the fourth attempt did not differ much in relation to earlier attempts for the same inhibitor.

#172

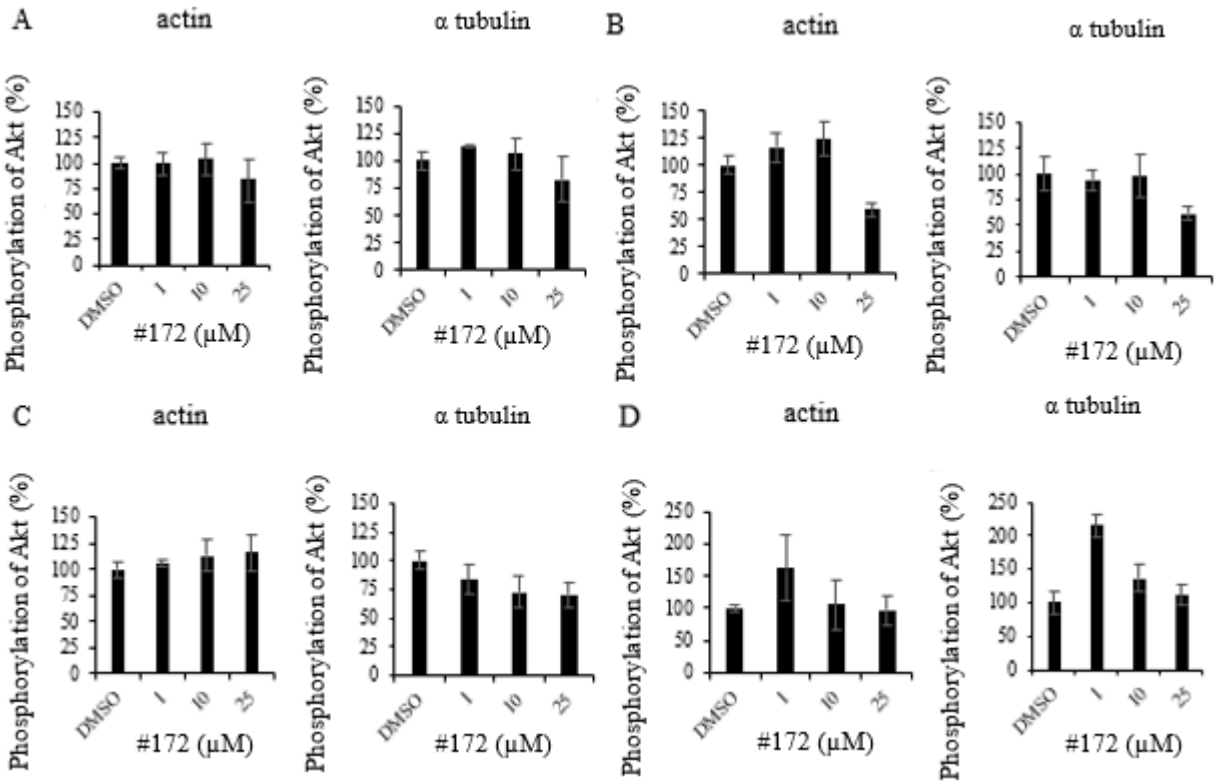
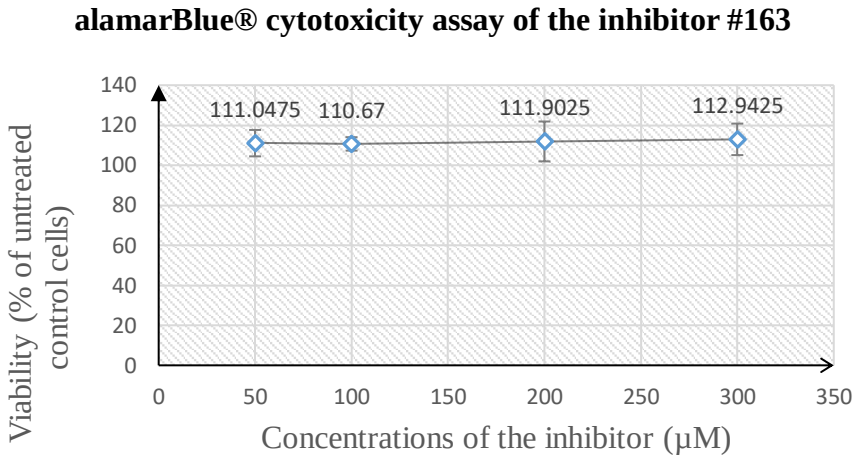


Figure 18. Effect of #172 on insulin-stimulated Akt phosphorylation in L6 myotubes. The level of Akt phosphorylation in myotubes treated with DMSO (vehicle) was set to 100% (control) (A, B, C and D indicate the graphs representing the four independent experimental trials each containing three repeats. The L6 myotube lysates were immunoblotted (not shown) against p-Akt or pan-Akt antibodies and the signal output was converted to percent activation by normalizing against house-keeping gene actin or α-tubulin. The values are the means ± relative standard deviation of three experimental repeats in each trial

5.2 Biological evaluation of inhibitors #163 and #170A using cell viability test

(1) #163



(2) #170A

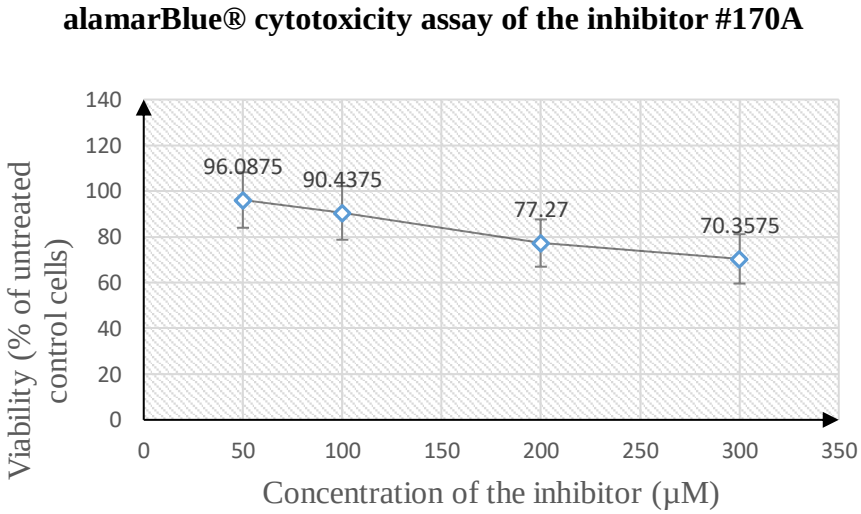


Figure 19. Analysis of alamarBlue® cytotoxicity assay of (1) #163 and (2) #170A. Graphs show the combined average results of the four different experimental trials for two different inhibitors on different days, where X-axis represents the four different concentrations 50, 100, 200, 300 (µM) of the inhibitors and Y-axis represents viability of L6 myotubes (% of control) after 20 hours of incubation with inhibitors. The error bars indicate relative standard deviation of the four different experimental trials at each concentration value.

Figure 19 (1) and (2) show the graphical analysis of alamarBlue® cytotoxicity assay of the inhibitors #163 and #170A that were selected for the assay out of the eight inhibitors studied in the present thesis. In total, four experimental trials were carried out for both the inhibitors on different days.

L6 myotubes treated with the increasing concentrations of the inhibitor #163 proliferated above control level (>100%) indicating the inhibitor did not affect the growth of the cells negatively (Figure 19.1). The viability ranges from ~111% at 50 µM to ~113% at 300 µM suggesting that #163 is not toxic to myotubes. In Figure 19.2, the cell viability decreases with the increasing concentrations of the inhibitor #170A. The viability ranges from ~96% at 50 µM to ~70 % at 300µM (Figure 19.2). At 300 µM, the myotubes are not killed at least 50% of the initial population suggesting that the median lethal dose (LD50) of the inhibitor #170A is above 300 µM. Thus, the results indicate that since the LD50 values are greater than 300 µM for both the inhibitors, at the experimental doses selected for Akt activation in L6 myotubes, the inhibitors are not toxic to myotubes.

6. DISCUSSION

Insulin signaling pathway is one of the most interesting research areas for novel drug targets of T2DM associated with insulin resistance. Moreover, an association between a novel cluster of T2DM known as severe insulin resistant diabetes (SIRD) and diabetic kidney disease has been reported in a Swedish research, though the new classification has not yet been implemented into clinical use[49]. At mechanistic level, intracellular lipid phosphatase, SHIP2, negatively regulates PI3K-Akt mediated insulin signaling pathway by catalyzing the hydrolysis of PIP3 to PIP2 upstream of Akt, and hence inhibits its activation[63]. In dysregulated insulin signaling pathway, this may lead to disease conditions including insulin resistance and T2DM. Hence, inhibition of SHIP2 might prove to be a beneficial treatment strategy for insulin resistance and T2DM over the traditional antidiabetic drugs such as ISA or insulin secretagogues.

Apart from the earlier genetic studies on the inhibition of SHIP2[63, 66, 68, 69], Suwa *et al* first time reported the discovery of novel small molecule inhibitor of SHIP2, AS1949490, by studying its inhibitory effects on the 5' phosphatase activity of SHIP2 in regulating insulin signaling pathway and glucose metabolism *in vitro* and *in vivo*[23]. Their immunoblotting results indicated an increase in insulin-induced Akt phosphorylation in L6 myotubes treated with AS1949490[24]. The *in vivo* analysis indicated that AS1949490 primarily inhibits SHIP2 activity in the mice liver. In the last decade after the report of the first SHIP2 inhibitor, AS19494190, several small molecule SHIP2 inhibitors have been reported in scientific literature[24,25,26,27,28]. However, they still lack drug like properties such as solubility, bioavailability, pharmacokinetics properties.

The present thesis focuses on the novel SHIP2 inhibitors that act mainly in skeletal muscle tissues since it represents the primary site of insulin resistance in CKD[102]. Inhibitors that function primarily in skeletal muscle may provide a new alternative to the current treatment strategies for the T2DM patients suffering from CKD[102,103]. In this thesis, I have attempted to study the capacity of eight novel small molecule SHIP2 inhibitors to increase the phosphorylation of Akt using immunoblotting. Based on the results of immunoblotting, further I carried out cytotoxicity assay of the two selected inhibitors, #163 and #170A.

The treatment of serum-starved L6 myotubes with SHIP2 inhibitors followed by insulin stimulation led to the phosphorylation of Akt, which is in line with the previous reports that inhibition of SHIP2 activity activates insulin signaling pathway[61, 62, 63]. I normalized the Akt phosphorylation to two different house-keeping proteins, actin or α-tubulin. Akt plays a role in the regulation of actin organization in cytoskeleton[104]. Moreover, interpreting the data using a single housekeeping protein

actin might provide a pseudo-positive or false negative activity of Akt since house keeping proteins are not always very stable under experimental conditions. Besides unstable control genes, uneven differentiation of L6 myotubes could also contribute to the varied fold changes in Akt phosphorylation in separate trials.

In 6 inhibitors out of the 8 inhibitors tested, the fold changes in Akt phosphorylation could not be repeated. The reason for this could be that the myoblasts were not seeded evenly in 12 well plates in some experiments. It is to be noted that the higher the passage number, the more difficult it is to differentiate L6 myoblasts, which indicates that in future we need to be more careful while differentiating L6 myoblasts and should keep the track of timings and circadian rhythm of this immortalized cell line[105] during splitting. In L6 myotubes treated with inhibitor #163, Akt phosphorylation increased almost 3-fold or above in three separate trials out of four trials in concentration dependent manner upon insulin stimulation and peaked at 25 µM concentration of #163. I normalized Akt phosphorylation against α-tubulin house-keeping protein (data not shown) in addition to actin to screen the effect of experimental conditions and test proteins on loading control proteins to alleviate erroneous results. Interestingly, upon normalization against α-tubulin, the Akt activation decreased drastically at 25 µM contrary to its highest activity in case of actin. This variation might be due to the differential stabilization of microtubules and actin organization and cell motility by differential expression of Akt phosphorylation[104,106]. The variation in normalization values could also be due to an experimental error. The samples for 25 µM were loaded on the edge of the blot and might not have been soaked evenly during incubation in the antibody dilutions leading to uneven signals and thus false-reduced Akt phosphorylation in the case of α-tubulin at 25µM concentration of #163. Treatment of myotubes with #170A in trial A also increased Akt phosphorylation almost 2-fold at 25 µM of the inhibitor in concentration dependent manner. Though, the results could not be repeated in trial B and trial C of #170A. Moreover, similar fold changes in Akt phosphorylation in three trials of #163 and trial A of #170A was the impetus for their selection for alamarBlue® assay to examine their cytotoxicity.

The viability of the myotubes was found to be above 100% at all concentrations of the inhibitor #163 with ~1.13-fold increase at 300 µM. This could be due to the increased glucose metabolism of L6 myotubes following treatment with #163[26]. On contrary to the results of #163, the viability of L6 myotubes treated with different concentrations of #170A decreased in concentration dependent manner ranging from ~96% at 50 µM to ~70 % at 300µM. This shows that over the course of time, the conversion of resazurin to resorufin decreased and the innate metabolic activity and

viability of cells decreased. From this, it could be inferred that higher concentrations (above 50 µM) of the inhibitor #170A had toxic effects which might have triggered cell death or apoptosis, impaired cellular metabolism, necrosis or disruption of cellular membrane which reduced the RFU[107]. However, for both the inhibitors, the LC50 concentration is higher than the experimental doses (1µM, 10µM, 25µM). Also, from figure 19, it could be inferred that at experimental doses of the inhibitors used in this study, the inhibitors are not toxic.

In this thesis, I have first time reported the study of eight novel SHIP2 inhibitors #160, #161, #162, #163, #167B, #170A, #171 and #172 to analyze their capacity to activate Akt kinase following insulin stimulation, and based on it, further analyzed cell viability by alamarBlue® cytotoxicity assay. There are more inhibitors to be tested from the chemical library in addition to the inhibitors tested in this study. This study would be beneficial in analyzing further small molecule SHIP2 inhibitors and thus would be a preliminary step in the discovery of novel small molecule SHIP2 inhibitors for T2DM therapeutics.

7. CONCLUSION

The present study was based on the role of SHIP2 enzyme as a negative regulator of PI3K-Akt mediated insulin signaling pathway and its association with insulin resistance and T2DM. Since SHIP2 is a 5' phosphatase that hydrolyzes PIP3 to PIP2 and ultimately inhibits the phosphorylation of Akt and hence its activity leading to the dysregulation of insulin signaling, inhibitors of SHIP2 could be a beneficial therapeutic strategy for the treatment of T2DM. One of the aims of this thesis was to identify potential SHIP2 inhibitors by their capacity to phosphorylate Akt kinase indicating the activation of PI3K-Akt mediated insulin signaling pathway. The immunoblot results showed ~3-fold increase in Akt activation when myotubes were treated with inhibitor #163 and ~2-fold increase in Akt activation when myotubes were treated with #170A in concentration dependent manner and were therefore chosen further for cytotoxicity assay. The results of alamarBlue® cytotoxicity and cell viability assay showed that viability was above control levels when the myotubes were treated with #163 whereas it decreased in concentration dependent manner when treated with #170. This indicated that #163 did not have toxic effects on the myotubes while #170 did affect the viability of the myotubes. However, the LD_{50} value for #170 was greater than 300 µM which is above the experimental doses of the inhibitors. Thus, immunoblot and alamarBlue® cytotoxicity assay results of the present work indicate #163 and #170A to be the potential candidates out of the 8 tested SHIP2 inhibitors.

All the 8 inhibitors tested in the present work showed fold changes in the Akt activation in varied degrees in different experimental trials. However, due to the large experimental variations, in future, more trials need to be performed with these inhibitors including #163 and #170A to get consistent results which would allow combined statistical analysis. One of the factors behind the large experimental variations might be the difficulty in culturing and differentiating L6 myoblasts which could be eased by working with this cell line over a period.

Apart from these 8 inhibitors, more inhibitors from chemical library need to be tested to select the best SHIP2 inhibitors that are potential candidate for further analysis. This thesis first time reports eight novel SHIP2 inhibitors #160, #161, #162, #163, #167B, #170, #171, #172 and attempts to screen the best out of them. This could be a significant step in the discovery of new T2DM drugs for more efficient, cost effective and safe treatment of the disease with least contraindications.

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I have been very fortunate that during my thesis work I have not only met outstanding colleagues but great friends. I got opportunity to share scientific ideas and discuss experimental issues with Vincent, Hong, Zyndrune, Jette, Anita, Rim, Raphael and of course Laura, for which I am highly grateful. During the lab meetings, I got opportunity to present my data for which I received feedback from the lab members helping me to improve my presentation skills and analytical skills. I learned a lot about data analysis and critical thinking during the lab meetings which would always be helpful in my future projects. During the lab meetings, I deepened my knowledge of not only my project but also of the projects that other lab members worked on which provided me an opportunity to become aware of the ongoing projects in diabetes research.

I am also highly thankful to my father Kishorkumar Mehta, my mother Rama Mehta and my brother Chintan Mehta for their unconditional love and support in all aspects of my life whether in success or failure and I shall always be grateful of this privilege that my family has provided to me.

Saumya Mehta

Helsinki, December 2019

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